A METHOD FOR PATTERNING PROTEINS AND ITS APPLICATION TO STUDY THE GUIDANCE OF NEURITE OUTGROWTH

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This work is dedicated to my Mother and to Pachy.

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Declaration: The method called here "first procedure to pattern proteins" was developed by the bioelectronics group of The University of Glasgow.

CONTENTS

ABSTRACT

The work described in this thesis has consisted of two well differentiated parts. In the first part, a method for patterning proteins at a micrometer level over a glass surface was developed and assessed. This method was based upon the use of amino groups attached to certain parts of the glass surface, to define the areas to which proteins could be bound, and of a non-specific protein attached to the remaining parts of the surface, to define the areas that would not bind the specific protein. It was assessed with a variety of protein detection techniques, among them silver staining, detection of radiolabelled proteins, and enzymatic assays, and in the end it proved to be very effective.

In the second part, patterns of laminin were produced with this method, and these were used to culture nerve cells over them. The purpose of this was to see whether they would guide the processes produced by the nerve cells, and they actually proved to be able to guide them. Then, this patterning method was used to study the geometrical conditions that would have to be fulfilled by a laminin pattern that would guide this processes efficiently. The geometrical characteristics that were investigated were A): The maximum distance between separate laminin areas that the processes could ignore, B): The minimum width of laminin pathways that could be recognised by the processes, and C): The angles of the turns in the pathways that the processes could follow.

The results of these investigations yielded a number of insights into the mechanisms by which nerve growth cones find their way towards their targets, that can be sumarised as follows: A) In the case of the cell type studied here, i. e. chicken embryo dorsal root ganglion cells, the maximum width of a non-adsorbant stretch than can be crossed by its growth cones is around 20 μm. This figure correlates well with the length of the filopodia in those growth cones. B) The minimum width of a laminin line that a growth cone can recognize and follow is smaller than 1 μm. C) growth cones following a thin laminin line seem to travel faster than growth cones over unpatterned laminin. D) Growth cones can follow laminin pathways with turns as sharp as 30°. E) However, the sharper the angles are, the longer the time that growth cones take in turning through them.

FIRST PART:

PROTEIN PATTERNING

INTRODUCTION

PROTEIN PATTERNING

This first part of this work is devoted to describing the development of a method for micropatterning proteins over glass surfaces. Such a method could have a number of applications in situations where it is important to have a protein immobilised on well defined areas of a surface. One such situation could be the development of small scale biosensors, in which a protein would form the sensing part. In this case, the protein would have to be positioned very accurately over some kind of microelectrode, and a failure to do so would lead to serious trouble during the calibration of the device.

Originally, a major part of the project that was to form the body of this thesis was the production of a calcium sensor. This sensor would rely on the immobilisation of a calcium-binding protein, such as calmodulin, over a gold microelectrode, so that changes in the concentration of calcium would be detected as changes in the potential on the surface of the electrode. Unfortunately, that particular kind of sensor is not feasible, because it is impossible to make the protein layer impermeable to the rest of ions in the medium, and thus changes in the concentration of calcium in the protein layer lead to changes in the concentration of other ions that approximately cancel any change in the potential.

Having failed to produce a calcium sensor, the method for patterning proteins was put to another use, one that also requires accurate positioning of proteins over a surface. This was the investigation of neurite guidance by patterns of extracellular

matrix proteins. This work was met with more successful results, and these are described in the second part of this thesis.

PHOTOLITHOGRAPHY

Photolithography is a technique routinely used in the electronics industry to manufacture high-density electronic circuits. It is based on the use of photoresists, which are radiation-sensitive organic monomeric or polymeric compounds in a phenolic resin solution. Radiation, usually UV light, elicits chemical changes in these compounds that render them more (positive resist) or less (negative resist) soluble in an appropriate solution, used as the developer (Pacanski and Lyerla, 1979). This property is used to produce any desired pattern over a given surface. The basic procedure is as follows. A thin layer of resist is deposited on the surface, then the surface is exposed to UV light through a mask with a print of the pattern, and then the areas of resist that have become more soluble are selectively eliminated by exposing them to developer. The limit of resolution of this technique is approximately 1 μm. Once the surface of the sample has a pattern of exposed and protected areas (protected by a layer of resist), the exposed areas can be modified, so that when the rest of the resist is removed, the pattern of modified and unmodified areas remains. In electronics laboratories they usually use silicon wafers, and the modifications introduced into them are oxidations or other processes that change the conductivity of the sample; in this work, this technique has been used to produce patterns of proteins on glass slides, and so the modifications have been chemical changes in the glass that would allow binding the proteins to it.

The modifications performed on the glass surfaces were of two different kinds. On the areas of the glass first exposed by removing resist with the developer, the purpose of the modification was to produce a surface that would not bind proteins. After this was made, the remaining resist would be dissolved away with acetone, and on the newly exposed glass a second kind of surface would be made, one to which proteins could be effectively bound. After this, the protein would be added with the expectation that it would attach only to the latter kind of surface.

The greatest problem encountered during the development of the method outlined above, was finding a surface that would not bind proteins. This was due to the tendency of proteins to adsorb to almost every surface. For this reason, a short discussion of protein adsorption will be given next.

PROTEIN ADSORPTION

Protein adsorption has been a great problem for researchers trying to develop artificial heart valves, implantable sensors, and in general any device that would have to be in contact with the blood of a patient for an extended period of time. The reason is that plasma proteins adsorbed to these devices can trigger the formation of thrombi that can be fatal to the patient. This has led to extensive research on the nature of protein adsorption, and to a search for materials to which proteins would not adsorb (Andrade and Hlady, 1986).

Proteins in solution adsorb to solid materials in contact with the solution whenever the free energy of the interface decreases with the adsorption. This means that if the protein can present

chemical groups to the solid surface that have more affinity with it than water molecules, while at the same time presenting hydrophilic groups to the water, it will get adsorbed. Due to the strongly amphipatic nature of proteins, most of them are capable of doing this when presented with any kind of surface. Thus, the adsorbed protein is bound to the surface by a fairly large number of weak, non-specific points of contact, that, together, form a strong linkage (Young, et al., 1988a, Young, et al., 1988b).

In general, protein adsorption is irreversible, in the sense that if the solution outside the surface is depleted of proteins, the amount of protein on the surface remains constant, because the large number of contacts of each protein with the surface make it very unlikely that all of them will come off at the same time (specially since the water molecules competing to occupy the sites in the surface have less affinity for them). This irreversibility applies only to the monolayer of proteins that is in contact with the surface, for when there is more than a monolayer coverage, all the layers but the first are relatively easy to desorb. A diagram of the dynamic state of a protein molecule adsorbed to a surface can be seen in fig. 1.1, adapted from the model by Lundström (Lundstrom and Elwing, 1990).

In spite of the irreversibility, adsorbed proteins can be desorbed if a solution containing surfactants or other proteins is put in contact with the surface, although experimental results show that desorption will never be complete: at most, 40% of the original protein monolayer will be displaced (Bale, et al., 1987, Rapoza and Horbett, 1990). The extent to which proteins can be desorbed depends largely on the nature of the surface to which it is adsorbed. A diagram of the mechanism by which a protein

displaces another from a surface can be seen in fig 1.2., again adapted from a model by Lundström (Lundstrom and Elwing, 1990).

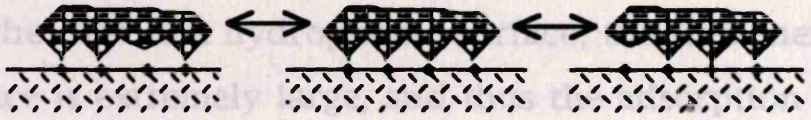


Fig. 1.1: A protein molecule is adsorbed to a surface due to the combined effect of many weak interactions. Each of these interactions breaks up very often, but they are re-established almost immediately. Only if they all were to break up at the same time would the protein desorb.

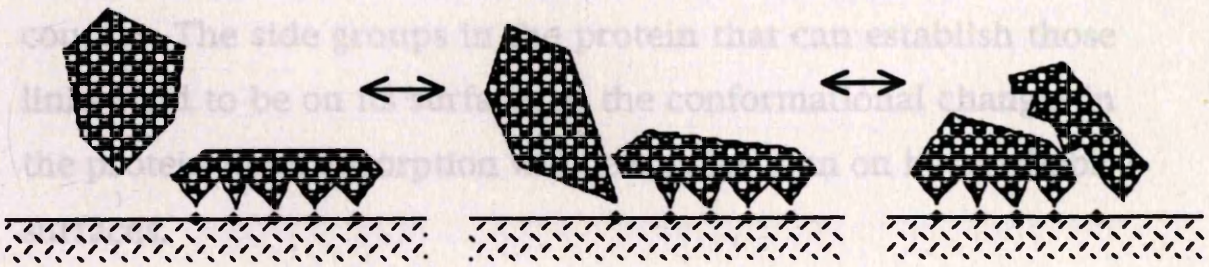


Fig. 1.2: One protein molecule adsorbed onto a surface can be displaced by another. This can happen because the displacing protein takes over one interaction site at a time, as its interaction with the first molecule breaks up, and thus the broken interaction can not be readily re-established. Obviously, this is a reversible process.

Depending on the nature of the surface, the side groups in the protein making contact with it will be more or less hydrophilic, and so the protein will have had to undergo more or less structural changes in order to be adsorbed. The strength of the adsorption varies, too: The adsorption will be stronger the larger the interfacial free energy between the water and the surface.

In the case of a hydrophobic surface, the free energy of the interface is extremely large, and thus the adsorption should be very strong. The hydrophobic chemical groups that will establish the contacts with the surface lie in the majority of the cases in the core of the protein, so it will have to change its conformation quite a lot to get adsorbed. On the other hand, on more hydrophilic surfaces the adsorption is going to be less strong, because the free energy of the interface between the surface and the water is smaller. The links between protein and surface are likely to be hydrogen bonds and, if the surface is charged, ionic couples. The side groups in the protein that can establish those links tend to be on its surface, so the conformational changes in the protein upon adsorption will be smaller than on hydrophobic surfaces.

This is confirmed by several studies. Bale et al. studied the ease of displacement by surfactants and the retention of activity of antibodies adsorbed to polystyrene copolymers of different hydrophobicities (Bale, et al., 1989). Although their results do not show a linear relationship, they nevertheless show a clear pattern: An increase in the hydrophilicity of the surface leads both to an increase in the ease of displacement of adsorbed proteins and to an increase in the retention of their biological activity when adsorbed.

Elwing et al. studied the conformational changes, amount, and ease of displacement by surfactants of the complement protein C3 adsorbed on modified silicon surfaces of varying hydrophilicity (Elwing, et al., 1988). Their results confirm as well what was outlined above, that is, that the more hydrophobic the surface is, the greater is the amount of protein that gets adsorbed, the more strongly it is attached, and the more its conformation changes in comparison with that of the protein in solution.

SURFACE MODIFICATIONS FOR PROTEIN PATTERNING

As has been pointed out above, in this study photolithography has been adapted to produce protein patterns. Different treatments are applied on different areas of the surfaces so that proteins bind to certain areas and avoid the rest. The surface to which proteins would be bound was made by treating the glass surface with aminomethylaminopropyltrimethoxysilane (aminosilane), a treatment that leaves glass covered all over in amino groups (the nature of the compounds and of the chemical reactions that take part in all the processes described here are explained in more detail later in this section). Over this surface, it was possible then to couple proteins using glutaraldehyde as cross-linker. Alternatively, proteins could simply be adsorbed to this surface, especially in the cases of proteins that retain their biological activity upon adsorption.

The non-adsorbing surface was, during the first stages of the work, made by treating the glass with dimethyldichlorosilane (chlorosilane), which renders a surface composed of methyl groups. Thus, the patterning procedure was composed of the

following steps (for a diagram, see fig. 1.3): first a pattern of resist on glass had to be made, then the exposed glass had to be covered with chlorosilane, the rest of the resist washed away with acetone, the newly exposed glass covered with aminosilane, and finally the protein had to be cross-linked to the amino groups on the glass slide with glutaraldehyde.

This procedure was later abandoned, for, as can be surmised from the above discussion on protein adsorption, chlorosilane does not make a good non-adsorbing surface. In any case, the method was not totally unsuccessful, and some of the results obtained using this compound are discussed below. This may be due to the fact that, although proteins do strongly adsorb to hydrophobic surfaces, most of them do so with enormous changes in conformation, so that they lose their biological activity. This is not an universal phenomenon, though, and some proteins do retain their activity.

In view of all this, it was decided that the next step in the project should be the search for a surface that would truly prevent protein adsorption. One material that is reported to produce such a surface is polystyrene modified with a series of Pluronic triblock copolymer surfactants $(PEO)_a(PPO)_b(PEO)_a$ (where PEO stands for polyethylene oxide and PPO for polypropylene oxide) (Lee and Ruckenstein, 1988). These copolymers adsorb to polystyrene and produce an interface between water and the hydrophobic polystyrene in which the hydrophobic block of PPO interacts strongly with the polystyrene while the hydrophilic blocks of PEO move freely in the water (Zhou and Chu, 1988, Tucker, et al., 1988). Proteins seem unable

to displace the copolymers from the surface, and so do not adsorb.

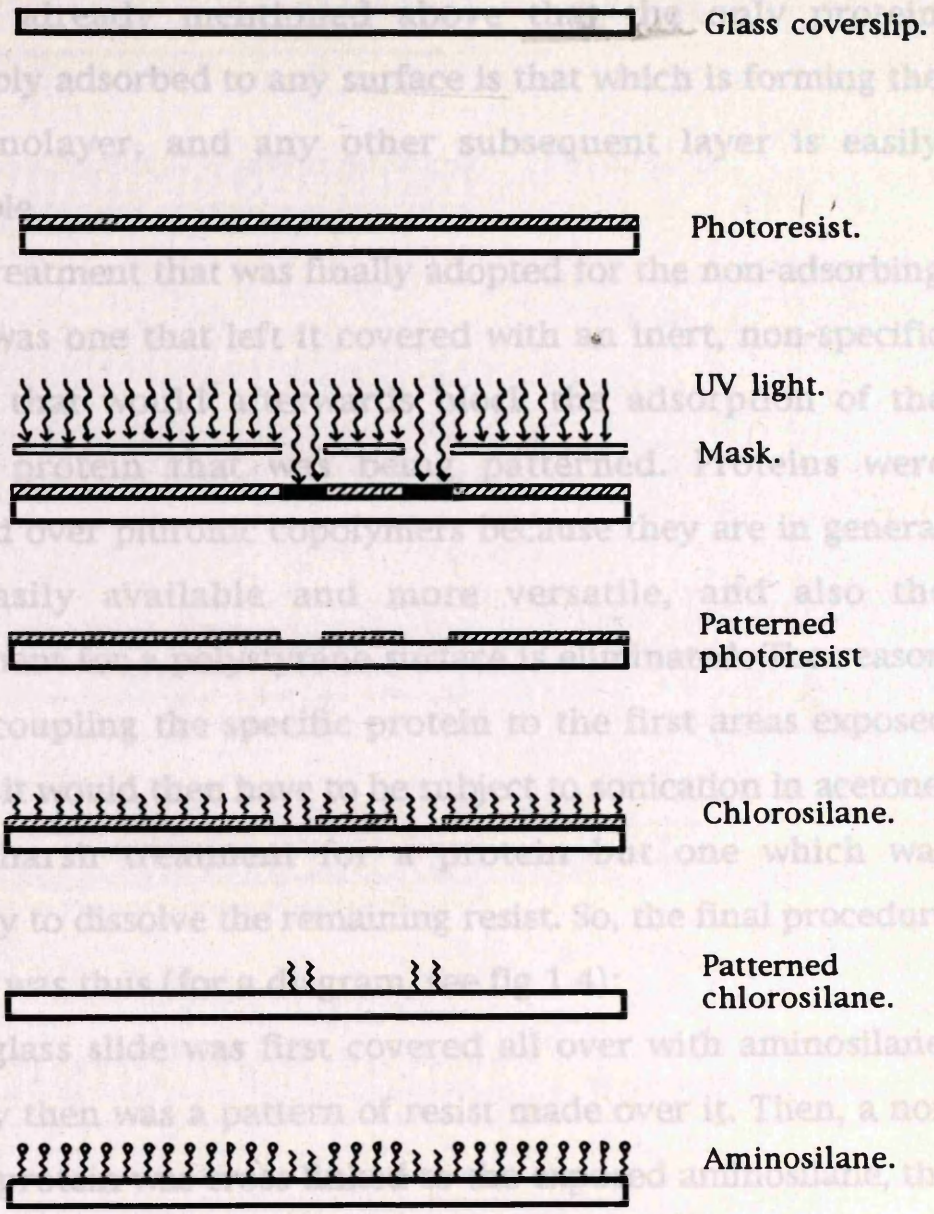


Fig 1.3: Diagram of the protein patterning method that relies on chlorosilane as non-adsorbing surface. See the text for details. After the last step depicted here, the slide would be incubated with the protein to be patterned.

Another type of surface that prevents protein adsorption is any surface already covered with proteins. This is obvious from the fact already mentioned above that the only protein irreversibly adsorbed to any surface is that which is forming the first monolayer, and any other subsequent layer is easily desorbable.

The treatment that was finally adopted for the non-adsorbing surface was one that left it covered with an inert, non-specific protein, that would afterwards block the adsorption of the specific protein that was being patterned. Proteins were preferred over pluronic copolymers because they are in general more easily available and more versatile, and also the requirement for a polystyrene surface is eliminated. The reason for not coupling the specific protein to the first areas exposed was that it would then have to be subject to sonication in acetone, a very harsh treatment for a protein but one which was necessary to dissolve the remaining resist. So, the final procedure adopted was thus (for a diagram, see fig 1.4):

The glass slide was first covered all over with aminosilane, and only then was a pattern of resist made over it. Then, a non specific protein was cross-linked to the exposed aminosilane, the resist was washed away, and the second, specific protein was coupled to the newly exposed aminosilane.

The next few pages will be devoted to introduce in some detail the main reagents that have been mentioned so far, and the reactions in which they are involved.

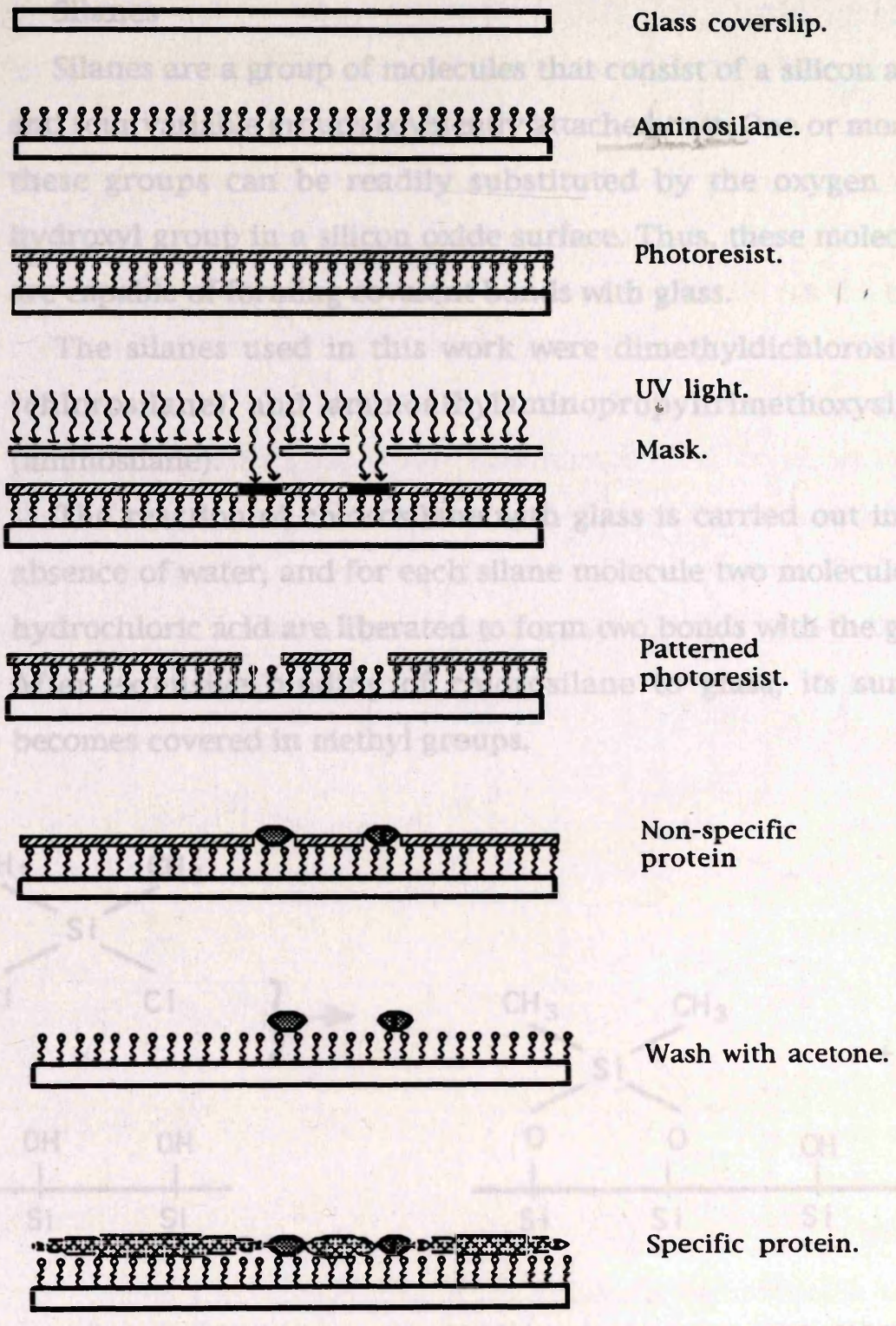


Fig 1.4: Diagram of the protein patterning method that relies on proteins to form the non-adsorbing surface. See text for details.

Silanes

Silanes are a group of molecules that consist of a silicon atom and four variable groups covalently attached to it. One or more of these groups can be readily substituted by the oxygen of a hydroxyl group in a silicon oxide surface. Thus, these molecules are capable of forming covalent bonds with glass.

The silanes used in this work were dimethyldichlorosilane (chlorosilane) and aminoethylaminopropyltrimethoxysilane (aminosilane).

The reaction of chlorosilane with glass is carried out in the absence of water, and for each silane molecule two molecules of hydrochloric acid are liberated to form two bonds with the glass. After extensive binding of chlorosilane to glass, its surface becomes covered in methyl groups.

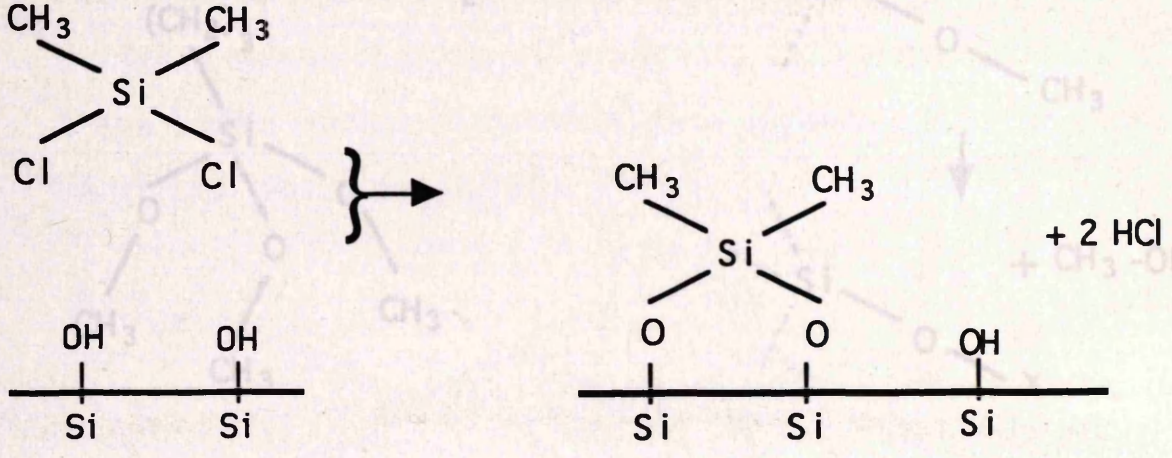


Fig 1.5: A molecule of chlorosilane reacts with SiOH groups on the glass surface, releasing two HCl molecules.

The reaction of aminosilane with glass is somewhat more complicated. It is carried out in the presence of water. Its three methoxy groups are the reactive groups, and they can undergo

three different reactions. Either they react with a hydroxyl group in the glass surface, establishing a bond with the glass; or they react with a water molecule, so that a hydroxyl group is left in their place; or they react with the hydroxyl group of a silane molecule that has undergone the second reaction described, establishing a bond with this other silane molecule (in the three cases, a methanol molecule is liberated) (Vankan, et al., 1988). It follows from this that the product of extensive reaction of aminosilane with glass is not a monomolecular layer, as in the case of chlorosilane, but probably a thicker layer in which the silane molecules are cross-linked to some extent. The most conspicuous groups exposed on the surface of the glass in this case are the amino groups.

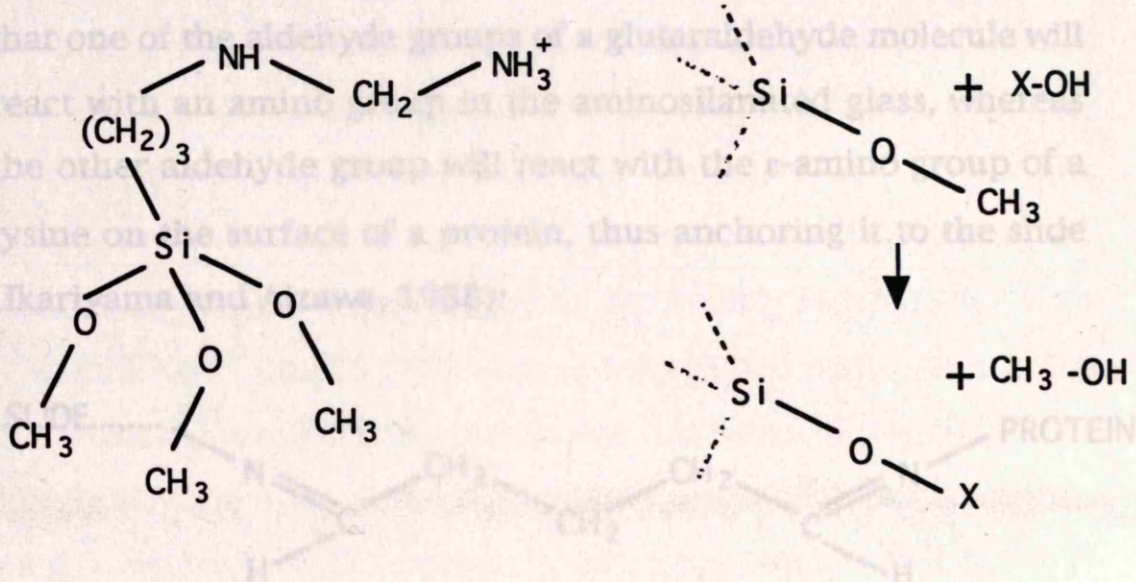


Fig 1.6: A diagram of an aminosilane molecule is depicted on the left. On the right, the reaction undergone by these molecule's methoxy groups, where X can be the glass surface, hydrogen (i.e., water), or another aminosilane molecule.

Glutaric acid dialdehyde

Glutaric acid dialdehyde, or glutaraldehyde (GA) is a small linear organic molecule with an aldehyde group at each of its two ends.

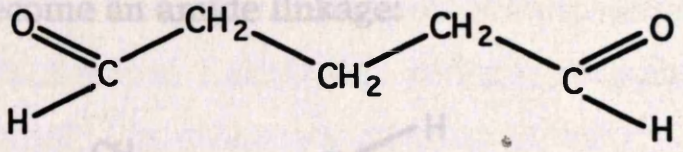


Fig 1.7: Diagram of a glutaraldehyde molecule.

Glutaraldehyde has been used in this work as a cross-linker, to attach proteins to the amino groups of the aminosilane on the glass slides. Aldehyde groups react with amino groups to form Schiff bases, liberating a water molecule. The basic idea, then, is that one of the aldehyde groups of a glutaraldehyde molecule will react with an amino group in the aminosilanated glass, whereas the other aldehyde group will react with the ε-amino group of a lysine on the surface of a protein, thus anchoring it to the slide (Ikariyama and Aizawa, 1988):

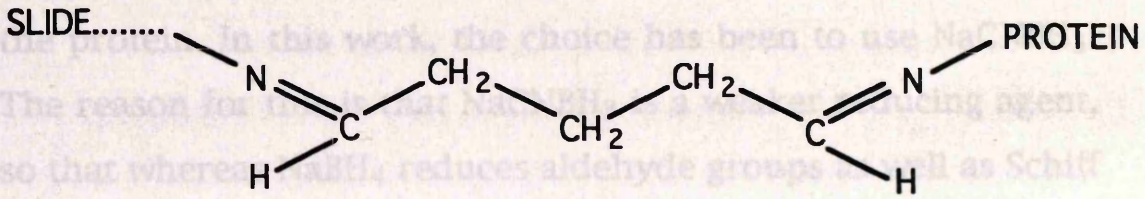


Fig 1.8: Diagram of a glutaraldehyde molecule after reacting with glass and a protein, coupled to both by Schiff bases.

Sodium Cyanoborohydride

Schiff bases are very unstable bonds, so for the purposes of this work it is preferable to stabilise them. One way of doing it is by reducing them with either sodium borohydride ($NaBH_4$) or with sodium cyanoborohydride ($NaCNBH_3$), in which case the bond would become an amide linkage:

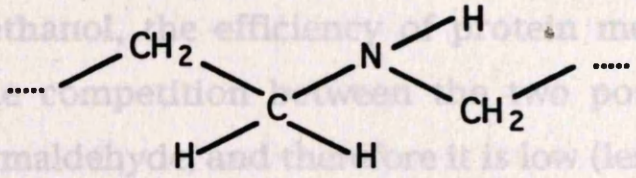


Fig 1.9: Diagram of an amide linkage.

This reaction is necessary both in the stabilisation of the linkage between the protein and the aminosilane and in the reductive methylation of the remaining amino groups of the protein, after it has been cross-linked with the glass (a step that will be discussed in the Methods section titled "a second procedure to pattern proteins"). Ikariyama et al. (Ikariyama and Aizawa, 1988) proposed incubating the aminosilanated slide with 100 mM $NaBH_4$ in PBS pH9, after it has reacted with GA and with the protein. In this work, the choice has been to use $NaCNBH_3$. The reason for this is that $NaCNBH_3$ is a weaker reducing agent, so that whereas $NaBH_4$ reduces aldehyde groups as well as Schiff bases, $NaCNBH_3$ only reduces Schiff bases (Jentoft and Dearborn, 1979). This characteristic provides the advantage that $NaCNBH_3$ and the protein can be added together, so that loss of GA-glass links during the incubation with the protein is avoided.

The convenience of using $NaCNBH_3$ instead of $NaBH_4$ can be seen if we consider the reductive methylation of the amino

groups of a protein. Traditionally, this was done incubating the proteins first with formaldehyde and then with $NaBH_4$. During such a treatment, the amino groups in the proteins (the ε-amino groups of the lysyl residues and the α-NH_2 terminus) form Schiff bases with the aldehyde groups in the formaldehyde, and afterwards $NaBH_4$ reduces the Schiff bases to form secondary and tertiary amines. Because $NaBH_4$ also reduces formaldehyde to produce methanol, the efficiency of protein methylation is a result of the competition between the two possible ways of reducing formaldehyde, and therefore it is low (less than 0.3 mol HCHO incorporated/mol amine in the best conditions). On the other hand, as $NaCNBH_3$ readily reduces Schiff bases but not aldehyde groups, the efficiency of protein methylation using $NaCNBH_3$ can be as high as 1.8 mol HCHO incorporated/mol amine (Jentoft and Dearborn, 1979, Jentoft and Dearborn, 1980). In addition, whereas $NaBH_4$ has to be used at high pH (about 9), $NaCNBH_3$ can be used at neutral pH.

Bovine Serum Albumin

Bovine serum albumin (BSA) has been used in the course of this work as a general model of proteins. This choice follows a long established trend that probably sprang out of the fact that it was isolated quite early from plasma in the pure form and that large amounts were easily prepared. Apart from that, the characteristics of albumin reflect well the characteristics of the majority of soluble globular proteins.

Serum albumin is found in the blood of almost all vertebrates, where it performs its functions. Its amino acid sequence is fairly well conserved across species, and for example 61% of the amino

acids are conserved among the sequences of rat and human albumin. It belongs to a multigene family of proteins that includes α-fetaprotein and vitamin D-binding protein.

The molecular weight of albumin is around 66 KDa. It has a very high solubility, probably due to the large amount of charged amino acid residues: around 200 at pH 7, out of a total of 585. Among them there are 56 lysines, that will play an important role in the course of this work. Apart from that, all the amino acids are present to some extent, the least abundant being tryptophan with just one residue per molecule. BSA is not glycosylated, and in that it is almost unique among plasma proteins. However, it contains one or two very tightly bound long chain free fatty acids per molecule when isolated from plasma.

The tertiary structure consists of three homologous globular domains, each of which is constituted by 10 α-helices. The three domains combine into a shape that is markedly asymmetric, and can be described as a solid equilateral triangle with sides of ≈ 80Å and average depth of ≈ 30Å. Altogether, about 67% of albumin is α-helical, and the rest is made up by turns or extended polypeptide. This structure is strongly stabilised by 17 disulphide bridges(He and Carter, 1992).

As stated earlier, albumin is a plasma protein, and it constitutes about one half of the total protein content in plasma. In addition, it is also found in tissues and bodily secretions throughout the body; the extravascular protein comprises 60% of the total albumin. Its most important function is the regulation of the colloid osmosis in blood, of which it is responsible for about 80% of it.

Albumin also functions as a transport protein, mainly of long chain free fatty acids. It has two very strong, five strong, and about twenty weak binding sites for fatty acids of the stearic acid type. The majority of these binding sites are in the form of non-specific hydrophobic pockets, while the ones with highest affinity combine these with electrostatic interactions. Apart from fatty acids, it binds and transports a number of other hydrophobic molecules, with a variable degree of affinity. Among them are bilirubin, which is toxic when free, some amino acids (notably tryptophan and cysteine), and a number of hormones, like thyroxine and the steroid hormones. In all these cases albumin acts as a back up transport protein, in the sense that all those compounds have more specialised transporters.

In the same way that it functions as a transporter, it acts as a detoxifier. Hydrophobic toxins of whatever source present in the blood, are bound by albumin and carried to the liver where they are excreted. It also has binding sites for toxic metals such as Cu^{2+} and Hg^{2+}.

A final function albumin fulfils is as a reserve source of nutritional protein. As such it has no transport system into the cells, but is first broken down to peptides and amino acids that can enter the cells and be used as appropriate.

A last remark on the functions of albumin is that none of them is irreplaceable: A few cases have been described of human patients with analbuminaemia whose plasma showed a high level of globulins and were capable of leading a perfectly normal life.

PROTEIN DETECTION

After devising the method of producing patterns of proteins on glass surfaces, it was important to find analytical techniques that would be capable of telling whether the method was effective, and to what extent it was so. Most proteins are transparent, and so a pattern made with a protein on glass is invisible. A technique had to be found which would render the protein visible in some way, and visible in very small amounts, even at the level of a monomolecular layer. A number of techniques were tried with a variable degree of success, and they will be introduced below.

FLUORESCENT LABELLING OF PROTEINS

A number of compounds, many of them organic compounds with aromatic structure, display the phenomenon known as fluorescence. This consists on the ability of the molecule to absorb a photon of a given wavelength, thus reaching an excited state, only to go back to its ground state by releasing another photon with a longer wavelength (the remaining energy being dissipated as heat). This characteristic can be used to detect those compounds at extremely low concentrations, by illuminating them with the appropriate wavelength and looking at them through a filter that only allows the passage of light with the characteristic emitted wavelength. In this way, the stained areas of the sample are seen brightly coloured against a dark background.

Fluorescent compounds are widely used in cell biology to label molecules, organelles, cells, etc., and to look at their distribution

in cells and tissues under the fluorescence microscope. The most commonly used microscope to visualise fluorescent probes is the epifluorescence microscope, in which the sample is illuminated by the same objective that collects the light that fluoresces back from the sample, so that almost the only light that travels towards the viewer is emitted fluorescence.

In this work fluorescent molecules were linked to the proteins prior to their patterning, so that the subsequent pattern would be fluorescent and thus visible under a epifluorescent microscope. The fluorochrome that was used was rhodamine isothiocyanate (RITC), which absorbs blue-green light and emits red light. The isothiocyanate moiety is a reactive group, and it reacts with the ε-amino groups of the lysines of proteins to produce a covalent link between the proteins and the rhodamine.

In this work, the RITC was used in solution. There are other methods described that are reported to be much faster, for example using RITC adsorbed to celite (Rinderknecht, 1962). Nevertheless, I obtained better results using the fluorochrome in solution, as described by Hudson (Hudson and Hay, 1989).

SILVER STAINING OF PROTEINS

Silver staining of proteins is a technique developed during the 80's mainly as a way to visualise proteins in polyacrylamide gels. It is an extremely sensitive technique, so much so that it is reported to detect down to 0.5 ng/mm^2 of protein in polyacrylamide gels (Sammons, et al., 1981). It relies on the formation of complexes between groups in the protein and diamine-silver salts which are dark brown in colour, although the exact mechanism by which it works is unknown. Glutaraldehyde

greatly enhances its sensitivity, and, when using it, the protein staining densities are almost linear with the molar percentage of lysine present. Some of the various methods that have been described are reported to be about 100-200 times more sensitive than those based on Coomassie Brilliant Blue (the classic way of staining proteins in polyacrylamide gels). The sensitivity of the technique varies to a certain extent with the nature of the protein, and for example it has been reported that it can not detect troponin C or calmodulin. Also, the silver stain has been found to be less sensitive with basic than with neutral proteins (Darbre, 1986).

The method that has been followed in this work is a modification of that of Porro et al. (Porro, et al., 1981). They devised the method for the staining of proteins in polyacrylamide gels. The modifications introduced in this work are intended to take into account the fact that, for proteins immobilised on a glass surface, there is no need to allow time for the solutions to diffuse into the vicinity of the proteins.

RADIOACTIVE LABELLING OF PROTEINS

Radioactive labels are among the most commonly used labels in the physical sciences, due to the fact that radioactive material can be detected in amounts smaller than any other kind of material. There are descriptions available of many methods of labelling proteins with different radioisotopes, the main ones being ^{125}I, ^{14}C, ^{3}H, ^{35}S. In this work proteins were labelled with both ^{3}H and ^{125}I prior to their inclusion in a pattern, so that it would be possible afterwards to detect the position of the radioactivity in the slide by taking an autoradiograph.

Labelling proteins with ^{3}H

Tritium is a radioisotope of hydrogen, which emits beta particles when it decays. Of all the beta emitters it is the least energetic (E_{max}=18.6 keV), and so the range of its radiation is very small (of the order of 0.3-0.4 mm in air). This gives it the advantage of providing very fine spatial resolution, which makes tritium the choice radioisotope to localise molecules inside cells. On the other hand, the low energy of its radiation makes it less easy to detect than other radioisotopes in small quantities.

The method that has been followed in the present work for labelling proteins with tritium involves the reductive methylation of ε-amino groups of lysines in the protein with formaldehyde and sodium $[^{3}H]_{3}$ cyanoborohydride, in an adaptation of the method described by Jentoft (Jentoft and Dearborn, 1979, Jentoft and Dearborn, 1980) (this has been already introduced in the section dealing with the immobilisation of proteins).

Labelling proteins with ^{125}I

^{125}I is a radionuclide that decays by electron capture, and its more significant radiation is in the form of γ–rays with an energy of 28 and 35 KeV. This radiation has a fairly long range, that depends on the nature of the medium through which it passes. As an example, only 50% of the energy is dissipated after travelling 2.5 cm through organic tissue. This means that, after labelling a sample with ^{125}I and taking an autoradiograph of it, it is not possible to detect fine detail of the distribution of the radioactive material, as was the case with ^{3}H. On the other hand, its higher energy emission ensures that much smaller amounts of material

can be detected, or, alternatively, that much less time is required to detect a given amount of material. For the completion of the present work, it effectively meant that the incubation of the sample with the X-ray film could be reduced from 3 months (labelling the protein with ^{3}H) down to 1 week (labelling the protein with ^{125}I).

Whereas other radionuclides used to label proteins (notably ^{3}H, ^{14}C and ^{35}S) are isotopes of elements that normally form part of the structure of proteins, iodine very rarely does so. The reason why it is widely used to label proteins is that iodine can easily be induced, with the aid of a catalyst, to substitute for a hydrogen in the side group of tyrosine, to form mono- or diiodotyrosine. Probably the most frequently used catalyst for this is Chloramine-T, which in aqueous solution, at about pH 7.5, becomes a mild oxidising agent that dissociates NaI and thus produces I^{+}, so that afterwards I^{+} gets readily incorporated into the fraction of tyrosine residues which are ionised.

In this work, instead of using Chloramine-T, a method first described by Markwell (Markwell, 1982) was followed. The method is based on the use of a new solid-state reagent marketed as Iodobeads™. The use of solid-state reagents has in general facilitated the use of radioiodine by simplifying the separation of iodinated products from the iodinating agent. This new reagent offers advantages over Chloramine-T in that it is stable under a larger variety of conditions and in that it can not be solubilised under any of the normal conditions of iodination. It consists of a chemical iodinating reagent (*N*-chloro-benzenesulfonamide) immobilised on 2.8mm diameter nonporous

polystyrene spheres, and the mechanism of action is essentially the same as that of Chloramine-T.

PEROXIDASE ASSAYS

Another method that was used to test the effectiveness of the patterning procedures consisted in producing patterns of horseradish peroxidase. This protein is an enzyme, and its enzymatic activity was assayed to test whether it was patterned.

Peroxidase is capable of oxidising a number of molecules using hydrogen peroxide as the supplier of oxygen radicals. One of these molecules is diaminobenzidine (DAB), which is soluble in water in its reduced form but highly insoluble in its oxidised form. Furthermore, The oxidised form is an intensely coloured molecule. So if immobilised peroxidase comes into contact with DAB and hydrogen peroxide under appropriate conditions, it will oxidise the DAB molecules, which will immediately precipitate in the near neighbourhood of the enzyme (Hudson and Hay, 1989). This reaction provides a very accurate way of determining the position of immobilised peroxidase (in fact it is often used in combination with electron microscopy), and was of course used in this work to prove the validity of the protein patterning procedure.

Peroxidase was also used to work out the amount of protein immobilised per unit of area. For this, an assay was performed which in essence is very similar to the one described above, but using aminoantipyrin instead of DAB as the reagent to be oxidised. Aminoantipyrin is soluble in water both in its oxidised and in its reduced form. The difference between one state and the other is that when reduced it is colourless, whereas when

oxidised it is pink. The speed at which known concentrations of peroxidase in solution oxidise a given concentration of antipyrin can be plotted against the concentration of the enzyme. Afterwards, the speed at which an unknown amount of peroxidase (in this case immobilised peroxidase) oxidises the same concentration of antipyrin can be interpolated in the plot, to work out the amount of enzyme present.

METHODS

PROTEIN PATTERNING

PHOTOLITHOGRAPHY

As was said in the introduction, two different procedures were devised to produce patterns of proteins. This section will start by describing in detail the procedure that was developed first. A diagram of it can be seen in fig. 1.3.

1) The glass coverslips on which the patterns were to be made were cleaned by immersing them in piranha solution (70% sulphuric acid and 30% hydrogen peroxide at 80°C) for 10 minutes, then rinsed with 15 changes of double distilled water (D.D.W.) and blown dry with a jet of clean air. Apart from cleaning the coverslips, this treatment maximises the hydroxyl group coverage of the surface of the glass, which is necessary for the subsequent silanation steps.

2) The clean glass coverslips were spin coated with S-1400-17 photoresist at 4000 rpm for 20 seconds. This produces a fairly thin layer (0.5 μm thick) of photoresist.

3) To remove the majority of the solvent from the layer of photoresist, the coverslips were baked at 90°C for 30 minutes in air.

Once baked, the coverslips with resist were ready to be exposed to UV light. Exposure causes the formation of carboxylic acid derivatives, which are much more soluble in developer than is unexposed resist:

4) The coverslips were exposed to UV light (350-400 nm, 15 mJ/cm^2) through a mask containing the desired pattern in tight contact with them

5) To remove the exposed resist, the coverslips were treated with 50% Microposit developer in D.D.W. for 75 seconds with gentle agitation, and then rinsed in D.D.W. and blown dry with clean air.

CHEMICAL DEFINITION OF THE SURFACES

At this stage, the coverslips were left with a pattern in resist. The next steps involved silanation, and broadly followed Kleinfeld's method (Kleinfeld, et al., 1988):

1) The coverslips with the pattern in resist were immersed for ten minutes in 10% dimethyldichlorosilane (chlorosilane) in chlorobenzene, and after that rinsed with pure chlorobenzene. This step is an improvement over Kleinfeld's, which took a much longer time (Britland, et al., 1992). The chlorosilane binds to the areas on the glass devoid of resist.

2) To get rid of all the remaining resist was somewhat difficult, due to the fact that the chlorobenzene hardens the resist. Usually, ultrasonication in acetone for 5 min plus exposure to UV light and developer was enough. The patterns over the coverslips were then in methyl groups.

3) The coverslips were then treated with 1% aminomethylaminopropyltrimethoxysilane (aminosilane) and 5% water in ethanol (pH5, adjusted with acetic acid) for 30 sec with continuous agitation, rinsed in one change of ethanol and baked at 120°C for 10 min. The aminosilane reacts with the areas of the glass coverslips that had not reacted with the chlorosilane, that is, the areas that were covered with resist before washing them with acetone, and as a result, there will be a pattern of amino and methyl groups on the slide.

PROTEIN IMMOBILISATION

The protein can at this stage be selectively cross-linked to the amino groups, using glutaraldehyde as cross-linker. The original method used for the immobilisation of proteins was taken from the work described by Ikariyama (Ikariyama and Aizawa, 1988). Basically, it consisted of incubating the coverslips with a glutaraldehyde solution for 1 hour, rinsing them, incubating them with the protein for another hour, rinsing them, and finally incubating them with $NaBH_4$.

One modification introduced in this procedure was to use $NaCNBH_3$ in conjunction with the protein instead of $NaBH_4$, as has been discussed in the introduction. Another question concerning the original procedure arose considering the incubation times. Ikariyama was working with a porous cellulose membrane, and recommended incubation times (both for the glutaraldehyde and the protein solution) of 1 hour. But, in the course of this work, no difference has been observed when the time has been reduced down to 5 minutes. This is presumably due to the fact that Ikariyama had to provide time for the glutaraldehyde and the protein to diffuse into the membrane, whereas in the present work contact between the reagents and the glass surface is immediate. The procedure, then, was modified as follows:

1) The aminosilanated coverslips were incubated with 2% GA in PBS for 5 minutes.

2) The coverslips were taken out of the GA solution and without allowing them to go dry, briefly rinsed in one change of PBS and then immediately transferred to the protein solution (25

μg/ml of protein in PBS pH7 plus 40 mM $NaCNBH_3$), in which they were left for another 5 minutes.

3) The coverslips were finally rinsed thoroughly with PBS.

A SECOND PROCEDURE TO PATTERN PROTEINS

The details of the second procedure to pattern proteins that was outlined in the introduction are described next (see fig. 1.4). Some of the steps of this procedure are identical to steps described above, and in these cases the reader will be referred to those described above.

1) The glass coverslips on which the patterns were to be produced were cleaned and then treated with aminosilane (as described earlier), so that their whole surface becomes covered in amino groups.

2) On top of the aminosilane was laid a layer of resist, then the coverslips were exposed to UV light through a mask and developed (all as described earlier).

3) At that stage there were areas of the coverslips covered with resist and areas with exposed amino groups. To these latter areas a protein (not the protein of interest, but an inert, non specific protein such as BSA) was cross-linked (again, the cross-linking method was described above).

4) The coverslips were then treated with 1% formaldehyde and 40 mM sodium cyanoborohydride in PBS for 1 hour, to block the ε-amino groups of lysines on the surface of the immobilised protein. This step is a variation of the glutaraldehyde cross-linking method, and its function was to avoid cross-linking of the protein that was to be added next to the amino groups of the first protein. The coverslips were then rinsed in five changes of D.D.W..

5) To remove the remaining resist, the coverslips were ultrasonicated in acetone until bright field microscopy showed that no trace was left (usually this took at least 2 hours).

6) The last step was to couple to the newly exposed amino groups the protein of interest, either with the method described earlier or by simple adsorption. If absorption was the chosen method, step four of this series could be left out, because in that case there would be no need of blocking the amino groups in the non-specific protein.

PROTEIN DETECTION

FLUORESCENCE LABELLING OF PROTEINS

BSA was labelled with rhodamine isothiocyanate (RITC), as described by Hudson (Hudson and Hay, 1989):

1) 0.05 mg of RITC per mg of protein were added to a solution of BSA of 2 mg/ml in a carbonate-bicarbonate buffer (pH8.5), and they were mixed overnight at 4°C.

2) The conjugated protein was separated from the free rhodamine by passing the mixture down a Sephadex column equilibrated with PBS 0.2 M.

SILVER STAINING OF PATTERNED PROTEINS

1) The slide with the protein pattern was incubated for two minutes in a diamino silver solution. To prepare the diamino silver solution, 1.4 ml of fresh NH_4OH and 21 ml of 0.36% NaOH were added together with vigorous agitation, and then 4 ml of

19.4% $AgNO_3$ were added to it slowly with continuous stirring, and finally distilled water was added to 100 ml.

2) after the two minutes, and without taking the diamine solution out, a few mls of developer (0.005% citric acid and 0.019% formaldehyde in distilled water) were poured over.

3) When the pattern appeared on the slide, it was taken out of the solution and immersed in 25% Amfix in distilled water for two or three minutes, and then rinsed in distilled water.

NOTE. Diamine silver solutions are potentially explosive and should not be stored. After use the discarded diamine should be destroyed by addition of HCl and diluted with water for disposal.

LABELLING OF PROTEINS WITH ^3H

The method that has been used in this work is an adaptation of that provided by Jentoft et al. (Jentoft and Dearborn, 1979). Jentoft et al. used [^{14}C]formaldehyde as the carrier of the radioisotope; in this case, $NaCNB[^3H]_3$ has been used instead. The detailed method is as follows:

1) The protein (1 mg/ml), HCHO (0.5 mM), and $NaCNB[^3H]_3$ (40 mM) were dissolved in 5 ml of 100 mM HEPES buffer (pH7.5), and the mixture was allowed to react for four hours at 37°C.

2) The mixture was dialysed exhaustively against 100 mM phosphate buffered saline (PBS).

LABELLING PROTEINS WITH ^{125}I

1) Two Iodobeads™ were added to 2 ml of 2 mg/ml BSA and 0.5 mCi $Na^{125}I$ in PBS, and the reaction was allowed to proceed for 40 min.

2) After this time, the reaction was stopped by removing the beads from the solution.

3) The beads were then washed twice with 1 ml PBS, and these washings were added to the solution with the protein.

4) This solution was passed through a G-10 Sephadex column equilibrated with PBS, and the protein was collected .

5) The protein was precipitated with 10% trichloroacetic acid (TCA), and the solution with the precipitate was spun at 3000 rpm for 10 min.

6) Finally, the pellet was resuspended in PBS, and the precipitation in TCA was repeated.

PEROXIDASE PATTERNS

Peroxidase was attached in several different ways (which will be described in the Results section) onto aminosilanated glass coverslips with a pattern of resist, and the enzymatic activity was assayed as follows:

1) The coverslips were rinsed with Tris buffer (50 mM Tris, HCl to pH 7.6).

2) Each coverslip was then incubated with 2 mls of 0.06% DAB in Tris buffer for 5 minutes at room temperature.

3) 4 ml of hydrogen peroxide were added to each coverslip, and the reaction was allowed to proceed for twenty minutes at room temperature.

4) The coverslips were rinsed thoroughly in tap water, allowed to dry and mounted.

DETERMINATION OF THE ACTIVITY OF IMMOBILISED PEROXIDASE PER UNIT OF AREA

First a standard curve was obtained in which the concentration of peroxidase was plotted against the change in absorbance at 377 nm of an aminoantipyrin solution:

1) A solution of 1 mM aminoantipyrin and 2 mM phenol in 50 mM Bis-Tris buffer, pH6, was prepared.

2) To 6 aliquots of 2 ml of that solution were added 0, 25, 50, 75, 100, and 125 µl of a 10 µg/ml peroxidase solution (in the same Bis-Tris buffer), to have a final concentration of peroxidase in each aliquot of 0, 0.25, 0.5, 0.75, 1, and 1.25 µg/ml.

3) The reaction was started by adding 10 µl of 25% hydrogen peroxide to each aliquot, and the reaction was allowed to take place for 8 hours at room temperature.

4) After that time, readings were taken with a spectrophotometer of the absorbance of each aliquot at 377 nm.

5) The concentration of peroxidase was plotted against the absorbance.

Once the standard curve was obtained, peroxidase immobilised on glass coverslips was assayed for its ability to oxidise aminoantipyrin, and the amount of enzyme present on each coverslip was determined from the curve:

6) Aminosilanated glass coverslips (22x22 mm^2) with peroxidase attached onto them were prepared in several different ways (see results), and rinsed thoroughly with D.D.W.

7) The peroxidase activity of the coverslips was assayed by adding 2 mls of the aminoantipyrin-phenol solution described above plus 10 µl of hydrogen peroxide to wells containing the

coverslips, and allowing the mixture to react overnight at room temperature.

8) The absorbance readings of the aliquots incubated with the coverslips were interpolated in the standard curve and so the amount of enzyme on each coverslip was worked out.

RESULTS

The results described in this section are those that came from the experiments performed to visualise and quantitate the proteins patterned on glass slides. As has been said earlier, two different patterning procedures have been developed. Some of the protein detection techniques were applied only to patterns that were made with either one of the procedures, and some were applied to patterns of both types. This is due to historical reasons, in the sense that it was not felt necessary to repeat detection techniques that had been employed in the past when the techniques being used at the moment were perfectly satisfactory. Nevertheless, the results of all the techniques are described here, as all of them throw some light on the patterns.

PATTERNS OF FLUORESCENTLY LABELLED PROTEINS

In order to visualise the patterns, the proteins that were to be patterned were conjugated to fluorochromes prior to their attachment to the slides, and then the slides were observed with an epifluorescence microscope. These experiments were performed before the second method for patterning proteins was developed, so these results apply only to the first method described in the methods section.

The fluorochrome used was rhodamine isothiocyanate (RITC), and the protein labelled was BSA. The reaction was carried out with the RITC in solution, and the separation of the protein from the unreacted fluorochrome was achieved by molecular exclusion chromatography. Most of the protein was recovered, and the fluorochrome to protein ratio obtained was of the order of 6:1, which was quite acceptable for the purposes of this work. It was this protein that was used to make patterns.

The patterns made using proteins so labelled were observed in an epifluorescence microscope. As shown in fig. 1.10, with this technique the patterns were clearly visible to their smallest detail, which means a resolution of about 1 μm. The problem with these results was that the methylated areas of the pattern showed fluorescence as well (although weaker). This fluorescence had a longer mean time of fading than the one in the amino areas, indicating that the fluorescent molecules were somehow different in each case, or that they were in different environments.

SILVER STAINING OF PROTEIN PATTERNS

Silver staining is a very sensitive method for detection of proteins. It was used in this work to visualise protein patterns made following only the first procedure described in the Methods section. The result was a very clear view of the pattern, as shown in fig. 1.11. This kind of result was obtained when the slide was taken out of the developer and put into the fixer at the exact moment at which the pattern became visible. If the slide was left for longer immersed in the developer, after 5 to 10 seconds the brown colouring would extend into the chlorosilanated areas, where no protein was supposed to be.

Another observation that was made was that, once the slides had been fixed, after a variable period of time (from 15 to 30 minutes) the coloured pattern would start to fade and eventually it would disappear.

As has been described in the methods section, the silver staining method involves glutaraldehyde, and indeed it is quite possible that the mechanism of staining is in some cases based on

Fig. 1.10: Epifluorecscence micrograph of a pattern of BSA labelled with rhodamine, which emits fluorescence in the red area of the spectrum. This pattern was produced with the first method described in the Methods section. The stripes are 10μm wide.

Fig. 1.11: Micrograph of a silver stained pattern of BSA taken with Nomarski optics. This pattern was produced with the first method described in the Methods section. The thinnest stripe is 1.5 μm wide.

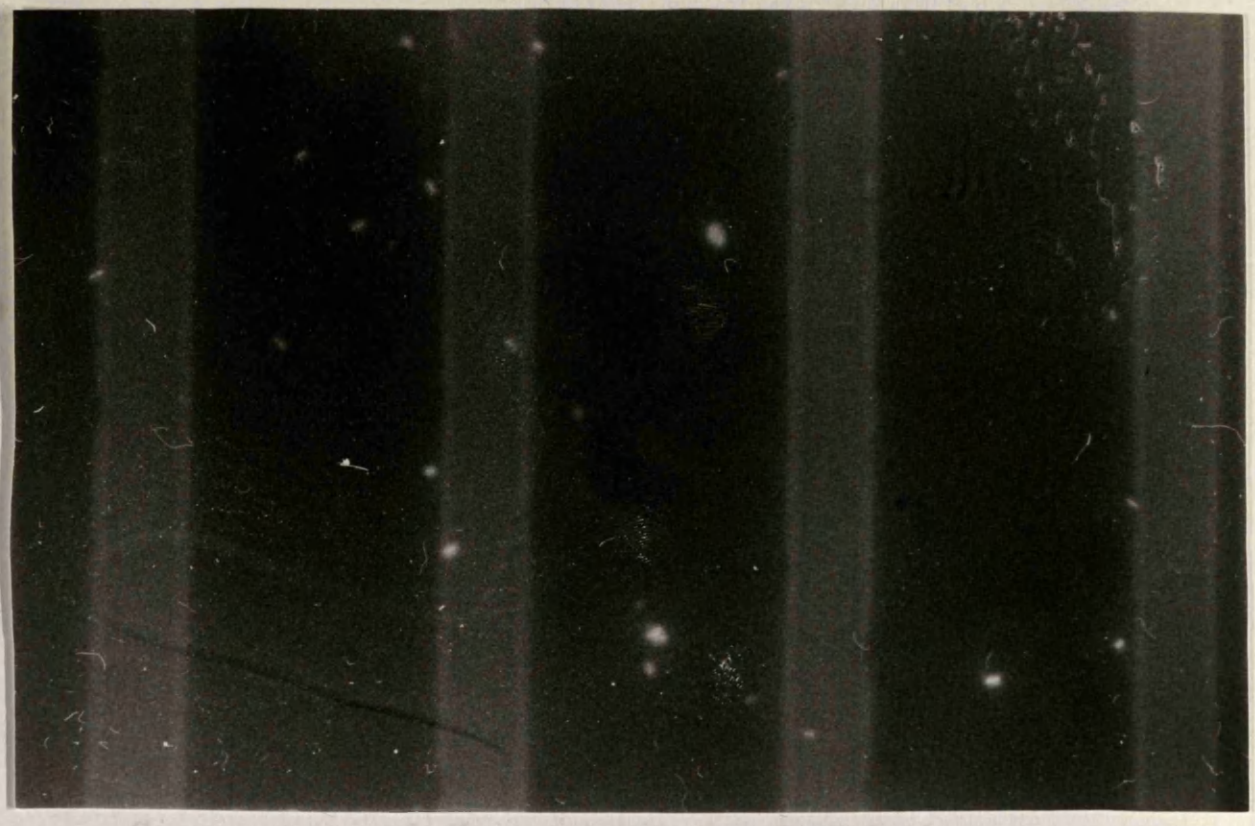


Fig. 1.10

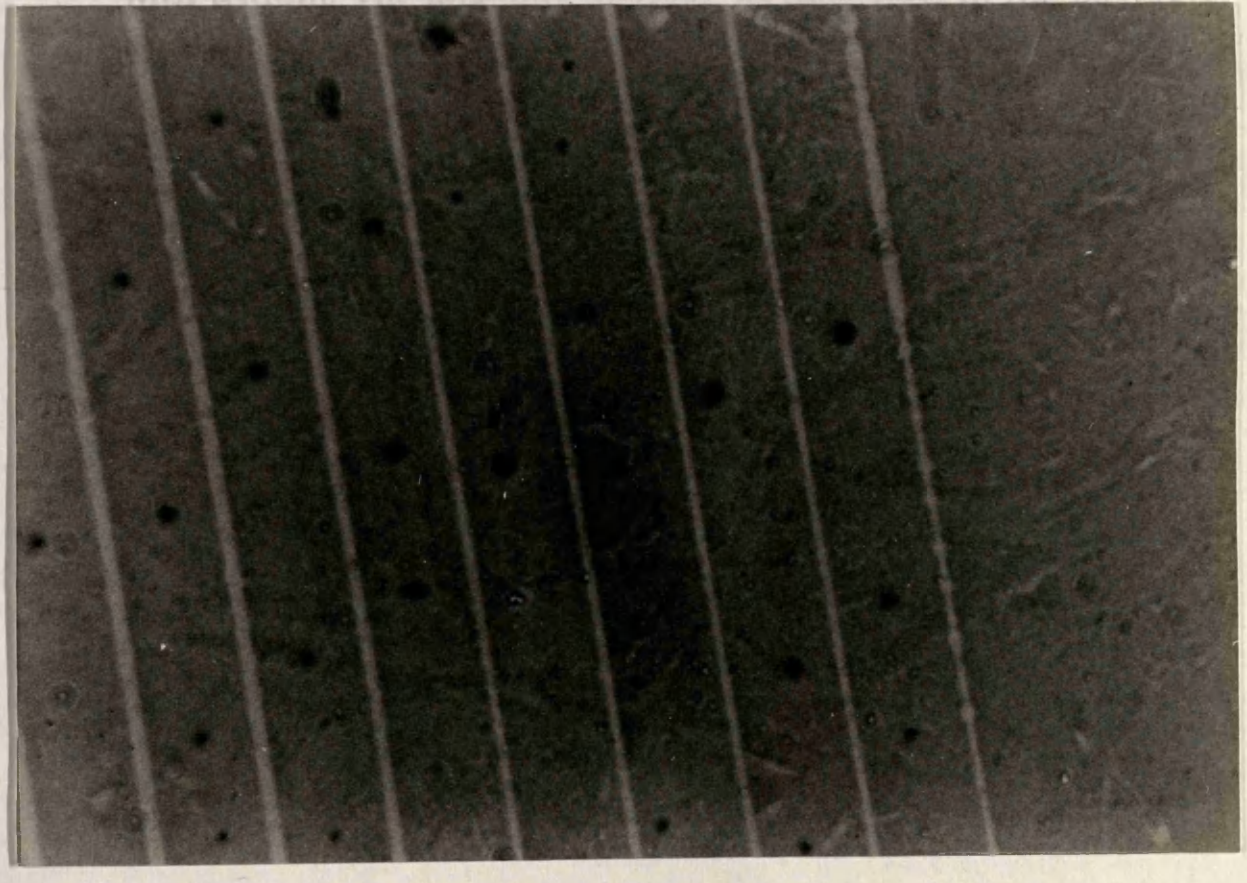


Fig. 1.11

complexes between the silver salts and the aldehyde groups. This posed a problem, since the patterning procedure involves glutaraldehyde. To test whether it was just the glutaraldehyde molecules that were being visualised with this technique, some coverslips were stained after the addition of glutaraldehyde but before the addition of protein. These coverslips also showed a brown coloured pattern after staining. Nevertheless, in this case the colour was much less strong, and it started to fade sooner than the slides with protein, about five minutes after fixing them.

PATTERNS OF ^{3}H LABELLED PROTEINS

After the inconclusive results of the methods involving fluorescence and silver staining, a new method was tried, which involved patterning proteins that had been previously labelled with tritium. The labelling method, as described in the methods section, involved reductive methylation of the lysines in the protein.

BSA was labelled in this way, and the yield was 5 mgs of protein with a specific activity of 0.51 MBq/mg. This [^{3}H]BSA was patterned following the first method described in the methods section (the second method had not yet been developed). The localisation of the radioactivity in the slides was shown by autoradiography, exposing the slides to X-ray film at -70°C for 8 weeks.

The films showed that the localisation of the tritium was higher in the areas of the slide covered with amino groups (see fig. 1.12.). This result was the most convincing proof so far that the proteins were effectively patterned. However, as shown in fig. 1.12, in some areas of the slide the pattern was erased or

Fig 1.12: Print of an autoradiograph from a glass slide with a pattern of BSA labelled with tritium. This pattern was made acording to the first method described in the Methods section. The pattern used here, as in figures 1.13 and 1.14, consisted of 6 macroscopic squares, the dimensions of which can be appreciated specially in fig. 1.14. The slide can be seen in its whole width (which is 2.5 cm), and the background radioactivity can be appreciated at the sides of the slide (in this case, only at the right hand-side).

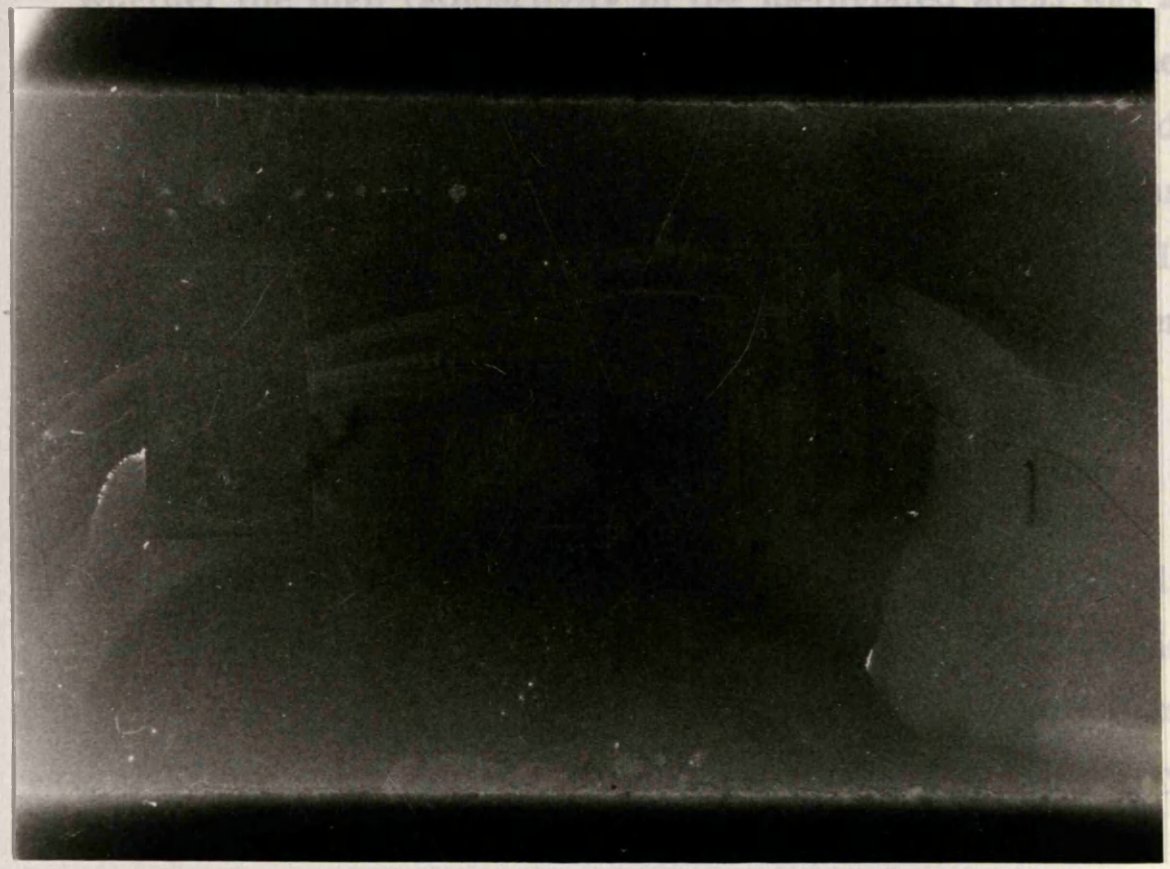


Fig. 1.12

unclear, and the background radioactivity in the areas covered with methyl groups was high compared to the general background.

It was necessary at this point to find out whether it was an unavoidable effect of the technique that the pattern was clearer in some areas than in others. It was necessary as well to find out whether the high radioactivity in the methylated areas was a constant feature when using this technique. So, to follow this line of visualisation, but to avoid the long exposure time required with ^{3}H, further experiments were carried out using proteins labelled with ^{125}I instead. As explained in the introduction, ^{125}I, being a more energetic radioisotope than ^{3}H, requires shorter exposure times, but gives poorer resolution.

PATTERNS OF ^{125}I LABELLED PROTEINS

BSA was labelled with radioiodine as described in the methods section, and 3.6 mg of protein were obtained with an specific activity of 0.568 MBq/mg. This protein was then patterned onto glass slides, and the slides were autoradiographed with X-ray film.

The protein was patterned first following the first described method in the Methods section. As can be seen in fig. 1.13, the radioactivity present in the areas of the slide covered with aminosilane was in average greater than that present over chlorosilane. However, the radioactivity over the chlorosilanated glass was still high compared to the general background, and its distribution was far from homogeneous. It was fairly clear then that some BSA was being non-specifically adsorbed to the hydrophobic parts of the slide. In an attempt to eliminate this

Fig. 1.13: Prints of autoradiographs from glass slides with patterns of radioiodine labelled BSA. This patterns were made acording to the first method described in the methods section. The background radioactivity can be appreciated at the sides of the slides. After the pattern had been made, each slide was washed with a different solution: **1.13 A** was washed with PBS, **B** was washed with 8M Urea, and **C** was ultrasonicated in 1% SDS.

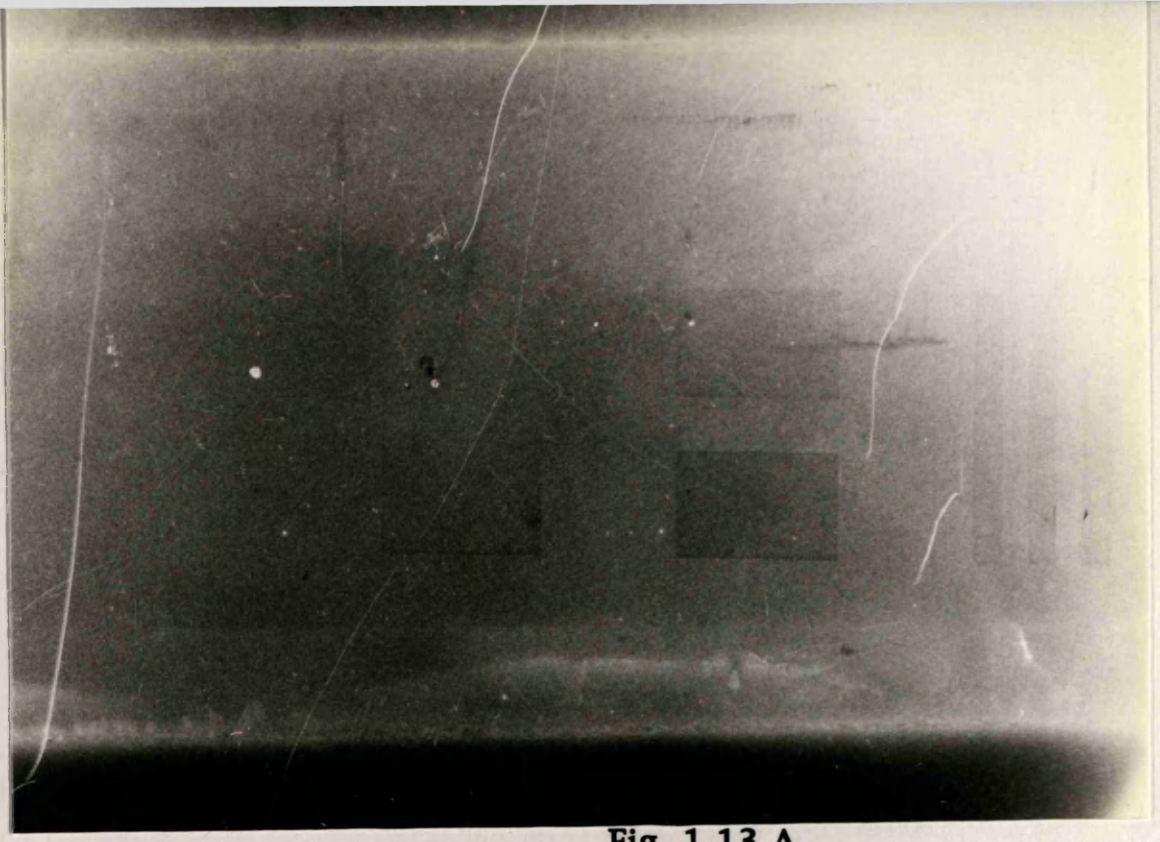


Fig. 1.13 A

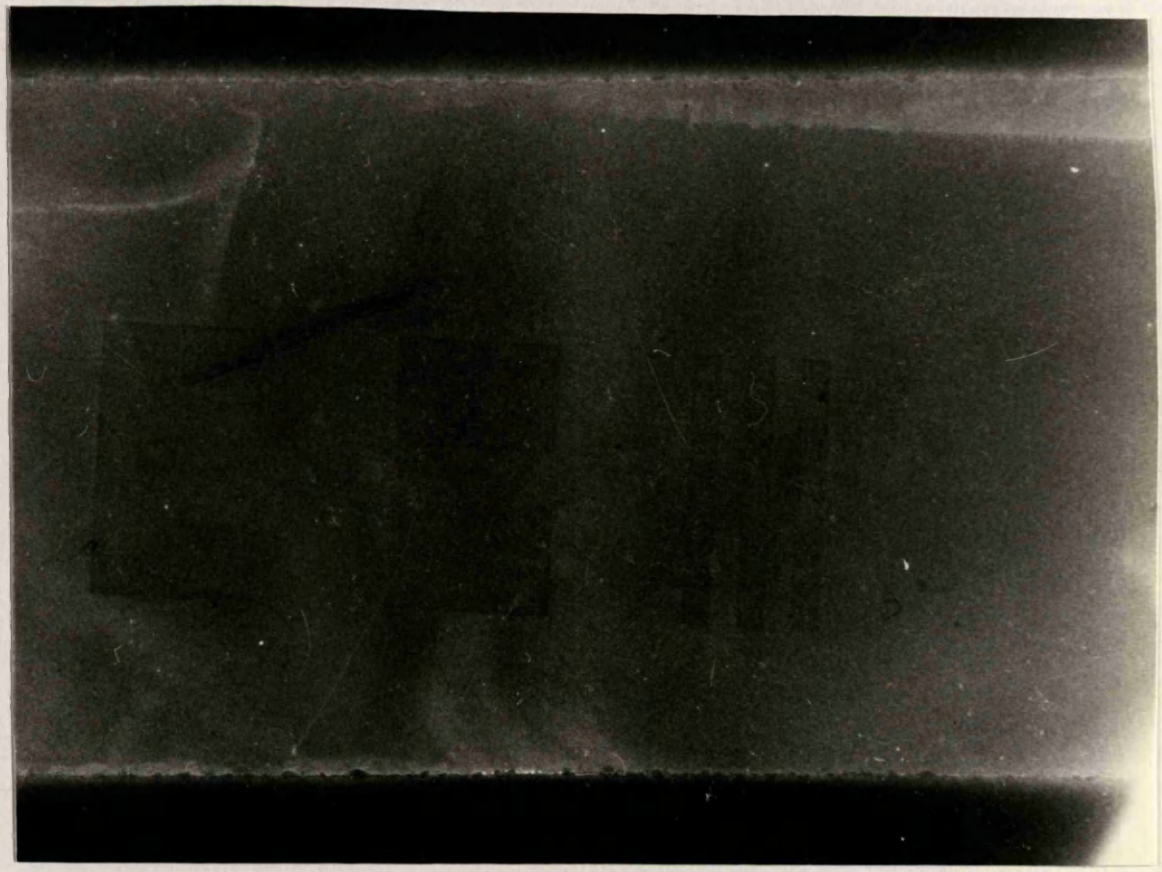


Fig. 1.13 B

Fig. 1.14: Print of an autoradiograph from a slide with a pattern of BSA labelled with radioiodine. This pattern was produced following the second method described in the methods section. Note that the background radioactivity, visible at the sides of the slide, is identical to the radioactivity in the patterned areas. The width of the slide is 2.5 cm.

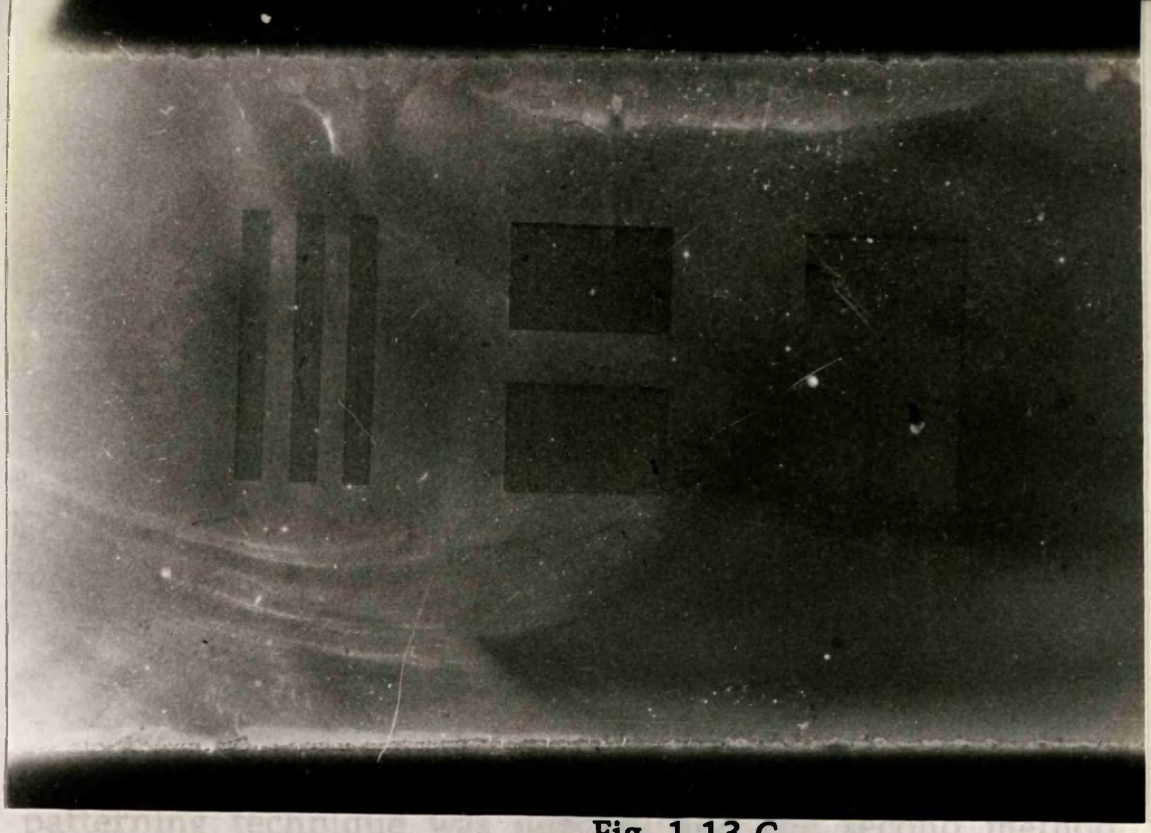


Fig. 1.13 C

Fig. 1.14

adsorbed protein, the slides were washed after patterning, using solutions and conditions that were expected to solubilise the protein to a certain extent: 8M urea for two hours, and 1% SDS ultrasonicating for four minutes. In both cases, the amount of protein adsorbed to the hydrophobic surfaces was still high, not much less than before (see fig. 1.13). The washing that gave a clearest pattern was that in which the slide had been sonicated in SDS, whereas the other treatment did not seem to affect much the amount of protein adsorbed to the chlorosilane.

It was at this stage that it was felt that a more reliable patterning technique was needed, and the second method described in the Methods section was designed. When this method was tried with [^{125}I]BSA, and an autoradiograph was taken, the picture that emerged was the best so far. As shown in fig. 1.14, that method gives a very clear view of the pattern, with very little background radioactivity in the parts of the slide where the first protein was attached, and high, homogeneous coverage of radioactivity in the areas where the second, radioactively labelled protein was meant to be attached.

PEROXIDASE PATTERNS

Several aminosilanated coverslips with a resist pattern were treated with peroxidase, as explained the methods section. The different treatments were the following (the details of each treatment are identical to those described the methods section for protein patterning), and each treatment was tested on 4 different occassions:

A) The peroxidase was cross-linked to the amino groups on the glass with glutaraldehyde and $NaCNBH_3$, and afterwards the resist was washed off with acetone.

B) BSA, instead of peroxidase, was cross-linked to the amino groups on the glass with glutaraldehyde and $NaCNBH_3$, and afterwards the resist was washed off ultrasonicating in acetone.

C) BSA was cross-linked to the amino groups on the glass with glutaraldehyde and $NaCNBH_3$, then the coverslip was treated with formaldehyde and $NaCNBH_3$, and ultrasonicated in acetone. After that, peroxidase was cross-linked to the remaining amino groups with glutaraldehyde and $NaCNBH_3$, and the coverslip was rinsed in PBS.

D) finally, peroxidase was cross-linked with glutaraldehyde and $NaCNBH_3$ onto a coverslip with a pattern of aminosilane and chlorosilane, and rinsed in PBS.

All these different samples were assayed for peroxidase activity with the diaminobenzidine method. The results were:

Samples of the type A and B did not stain at all with DAB.

Samples of the type D stained with DAB, but not homogeneously, and the pattern was not discernible (not shown).

Samples of the type C, on the contrary, were stained with DAB only in the areas that had been covered with resist at the time when BSA had been cross-linked to them, so that the pattern on the coverslips was clearly visible. Fig. 1.15 shows details of this sample stained with diaminobenzidine.

Fig 1.15: Micrograph of a glass coverslip with a pattern of peroxidase, made following the second method, and stained with the diaminobenzidine assay. The light areas were covered with BSA, whereas the dark areas had peroxidase coupled to them. The sides of the square in the middle of the picture measure 200μm. Phase optics.

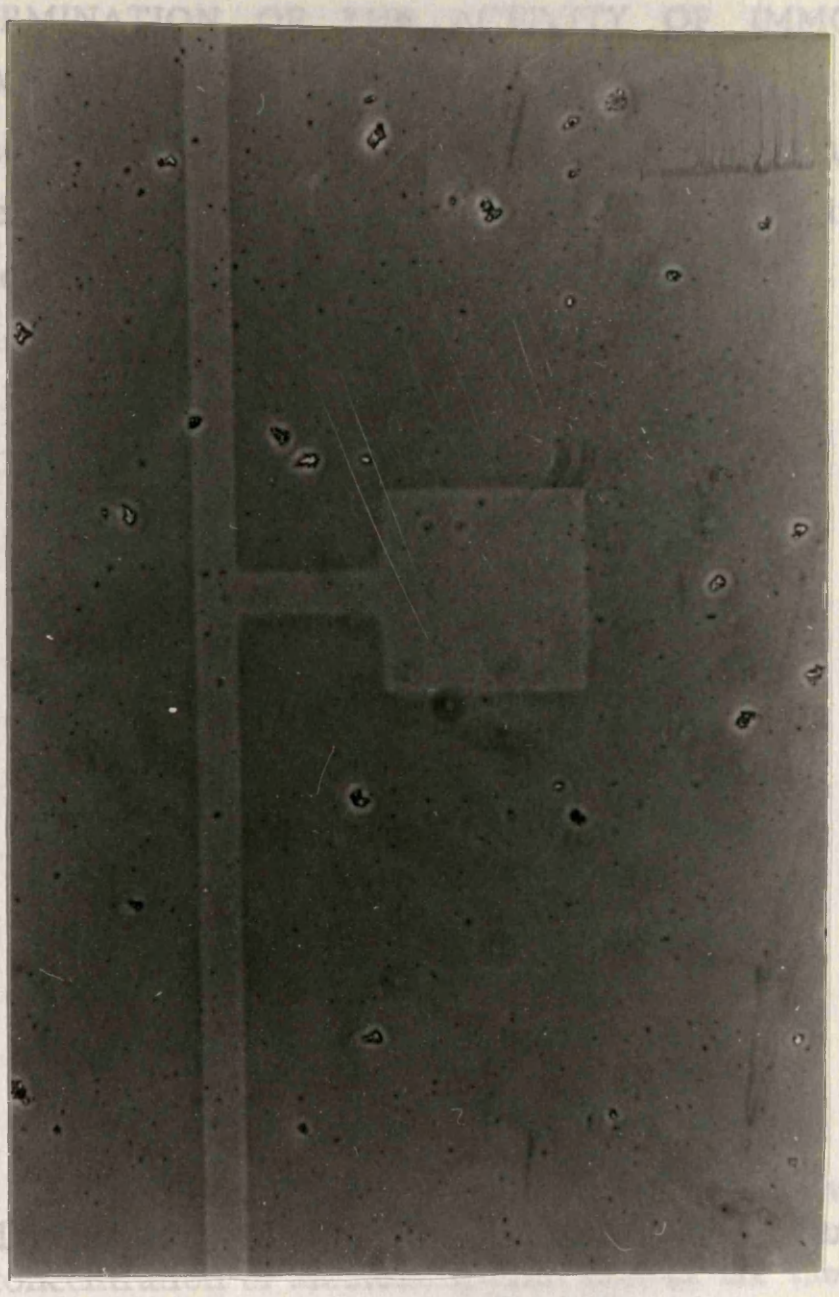
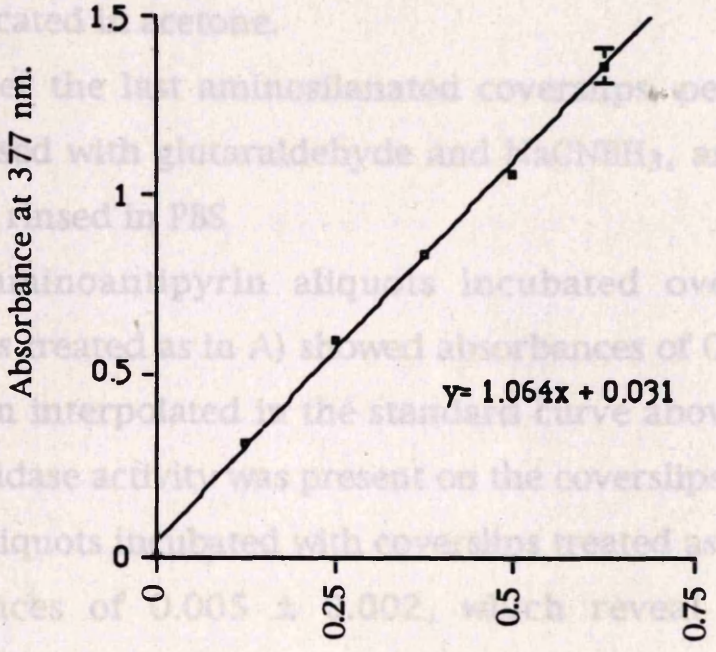


Fig. 1.15

DETERMINATION OF THE ACTIVITY OF IMMOBILISED PEROXIDASE PER UNIT OF AREA.

The standard curve that resulted from assaying known concentrations of peroxidase with aminoantipyrin-phenol solution can be seen in graph 1.1.



Graph 1.1: Curve showing the speed at which different concentrations of peroxidase oxidise a standard concentration of aminoantipyrin. (S.E.M.s are shown as error bars where they were greater than 0.02)

After preparing the standard curve, coverslips were assayed which had peroxidase attached to them in different ways. The different ways were as follows (the details of the procedures are the same as those described in the methods section dealing with protein patterning):

A) BSA was immobilised onto aminosilanated coverslips with glutaraldehyde and $NaCNBH_3$.

B) Coverslips with immobilised BSA (as in A) were incubated with formaldehyde and $NaCNBH_3$ and ultrasonicated in acetone, and then an attempt was made to cross-link peroxidase to them with glutaraldehyde and $NaCNBH_3$.

C) Over other aminosilanated coverslips, peroxidase was immobilised with glutaraldehyde and $NaCNBH_3$, and then ultrasonicated in acetone.

D) Over the last aminosilanated coverslips, peroxidase was immobilised with glutaraldehyde and $NaCNBH_3$, and then they were just rinsed in PBS

The aminoantipyrin aliquots incubated overnight with coverslips treated as in A) showed absorbances of 0.001 ± 0.002, that when interpolated in the standard curve above reveal that no peroxidase activity was present on the coverslips.

The aliquots incubated with coverslips treated as in B) showed absorbances of 0.005 ± 0.002, which reveal a negligible enzymatic activity.

The aliquots from coverslips C) showed absorbances reflecting a mean enzymatic activity corresponding to 76 ± 6 ng per coverslip, or about 16 ng/cm^2.

Finally, the aliquots from coverslips D) showed absorbances reflecting a mean enzymatic activity corresponding to 0.55 ± 0.04 μg per coverslip, or effectively about 0.12 $\mu g/cm^2$.

DISCUSSION

In this section the results described above will be discussed, and a case will be made that the attempt to pattern proteins on glass was largely successful. After that, other protein patterning methods will be discussed in comparison with the one described in this work, and the advantages and disadvantages of each one shall be highlighted.

PATTERNS OF FLUORESCENTLY LABELLED PROTEINS

After viewing patterned BSA labelled with rhodamine, it became clear that the fluorescence was not entirely restricted to the areas of the slide onto which BSA was supposed to be covalently bound. There were two possible explanations for this. The first was that in the methylated areas the fluorescence came from free rhodamine that had remained with the protein even after the separation step. This free rhodamine would bind to the methyl groups by hydrophobic interactions. The second explanation was that fluorescently labelled protein was being non-specifically adsorbed to the methylated areas.

Both explanations would agree with the fact that the fluorescence had a different fading speed in each kind of area: Free rhodamine would have a fairly different environment than coupled rhodamine, and adsorption of labelled protein would result in a different conformation of the protein than that resulting from cross-linking, thus providing again a different environment for the fluorochrome. It is to a large extent the environment of the fluorochrome that determines its fading speed.

The complete explanation was probably a combination of the two, especially in the case of BSA which has so called

hydrophobic pockets where uncoupled molecules of rhodamine could "hide" during the separation steps. In any case, to eliminate the possibility of free rhodamine interfering with the results, other labelling techniques were tried.

SILVER STAINING OF PROTEIN PATTERNS

Silver staining is an extremely sensitive technique for the detection of proteins, but the mechanism by which it detects them is very poorly understood. As was noted in the introduction, it may depend on the formation of silver complexes with amino groups, or amide groups, or aldehyde groups, or perhaps with more than one of them (Dion and Pomenti, 1983). It seems certain that at least aldehydes or aldehyde-amino complexes can take part in the staining process. This fact prevented this technique from giving a definitive answer to the question of whether the proteins are patterned or not. Nevertheless it gave interesting insights into the method.

As noted in the results section, staining patterns made only with glutaraldehyde showed that at least the glutaraldehyde was properly patterned. Furthermore, staining protein patterns (in this case, made with the first procedure described) gave some indication that the protein was properly coupled to the amino groups on the slide, because the staining was stronger and more permanent than that of the glutaraldehyde patterns.

On the other hand, when protein patterns were used, the methylated parts of the slide would go dark as well, only with some delay with respect to the aminated parts and with fainter staining. This effect could have several explanations. Assuming that the protein would adsorb to the methyl groups, the delay in

staining could be due to the different conformation in which the protein adopted in each case. At the same time, the harsh conditions imposed by the silver staining procedure (very high pH, mainly) could have washed away a large part of the adsorbed protein, but not of the covalently bound protein. Also, the fact that the protein on the aminosilane is cross-linked with glutaraldehyde could account for the stronger staining, for relatively large amounts of glutaraldehyde coupled to the amino groups on the glass could be partly responsible for the staining. A final explanation could be that there is no protein adsorbed to the chlorosilane, and the silver diamine salts crystallise on the hydrophobic surface using the protein in the aminosilanated surface as nucleation points. This last explanation is perhaps less convincing, but possible nevertheless.

PATTERNS OF ^{3}H LABELLED PROTEINS

Patterning BSA labelled with tritium (following the first procedure) gave results that largely agreed with the results of the previous experiments, to the effect that the radioactivity was higher in the aminosilane areas, but some was still present in the chlorosilane areas as well. In this case there was no question of free label adsorbing to the chlorosilane, because, on one hand, sodium cyanoborohydride (which was the carrier of tritium) is very water soluble, so that even a gentle washing would have removed it. On the other hand, this compound does not have any special affinity for the protein molecules (as opposed to RITC, that could be carried by BSA in its hydrophobic pockets), so it would have been totally separated from the protein in the molecular exclusion column. All these points suggest that the

radioactivity present in the coverslips corresponded entirely to labelled protein.

One effect that was noticeable from the radioactivity on the parts of the slide covered with chlorosilane, that is, from the non-specifically adsorbed protein, was that it was not evenly distributed. Whatever the explanation for that, it seemed clear that there was protein adsorbed to the hydrophobic areas. To confirm this, the work was pursued further with radioiodine-labelled proteins, which needed a much shorter time to produce an autoradiograph. In any case, with the protein adsorbing to the chlorosilane, the possibility of observing microscopic details of the pattern was lost.

PATTERNS OF ^{125}I LABELLED PROTEINS

The results of making patterns with [^{125}I] BSA confirmed the results obtained with tritium labelled BSA, that is, that there was a considerable amount of BSA being adsorbed to the chlorosilane, and that its distribution was quite heterogeneous in all the areas of the pattern. In this case, as in the case of tritium, it is quite certain that all the radioactivity comes from labelled protein, for much the same reasons.

After reaching this conclusion, I looked for some solution capable of washing away the non-specifically adsorbed protein. The solution had to be able to solubilise proteins without being too harsh to them, so that the damage that the solution would inflict on the protein cross-linked to the glass would be reversible. The ones that were tried were 8 M urea and 1% SDS, the latter both by itself and in combination with ultrasonication, and with plain PBS as a control. None of the solutions seemed to

differ markedly from PBS, perhaps only ultrasonication in SDS, and even that was not totally satisfactory.

At this point of the work it became apparent that the patterning procedure had to be refined, in the sense of finding a non-adsorbing surface to replace the chlorosilane. The surface that was chosen, as has already been mentioned, was aminosilanated glass with an inert, non-specific protein immobilised onto it.

When this new patterning method was tried with radioiodine labelled BSA, it was found, as described in the results section, that the radioactive material was restricted to the areas of the glass slide on which it was supposed to be, confirming the new method as successful for patterning proteins.

PEROXIDASE ASSAYS

The results of patterning peroxidase and assaying it with diaminobenzidine largely confirmed what had been found out with radiolabelled BSA, adding the extra dimension of showing not only physical presence but enzymatic activity.

The patterning method developed first gave a distribution of peroxidase activity that was quite similar to the distribution of radioactivity when patterning radiolabelled protein. The colour was fairly heterogeneously distributed all over the surface, and the boundaries between the two kinds of area were not discernible except in a few limited areas, haphazardly strewn over the coverslips.

The patterns made with the second method, on the other hand, showed quite clearly that the activity is confined to the

expected areas, closely restricted by the boundaries separating the two kinds of area and evenly distributed within them.

Another fact that transpires from this experiment is that the enzymatic activity of immobilised peroxidase is destroyed by ultrasonication in acetone. This was further shown in the experiments described next.

Peroxidase was used in another set of experiments aiming to quantitate the amount of protein present in each of the areas comprising the patterns. To this effect, it was attached to coverslips (without any patterning) in ways that mimicked the treatments that each part of a pattern undergoes during the second patterning procedure, and then its activity was measured and quantitated against the activity of known amounts of peroxidase in solution. This method is likely to underestimate the real amount of protein present on the glass, because the active centres of some molecules are likely to be sterically hindered by the immobilisation. In any case, it should provide information on the relative amounts present in the differently treated coverslips.

With this assay, the amount of peroxidase activity found in a cm^2 of immobilised peroxidase (this mimics the parts of the patterns that showed peroxidase activity with the diaminobenzidine assay) corresponded to the activity of 0.12 μg of peroxidase in solution. This figure can be translated as saying that there is roughly one active molecule per each 70 nm^2, a figure that would very nearly approach a monolayer coverage of the surface. This statement has to be viewed with caution, for, apart from the fact noted above that the assay measures activity and not actual amount of protein, the roughness of the surface is not being taken into account. Nevertheless, the figure reflects

other results reported in literature when using similar immobilisation techniques (Bhatia, et al., 1989, Meltzer and Silberberg, 1988).

By contrast, the activity detected on coverslips with immobilised BSA onto which attempts had been made to cross-link peroxidase, when interpolated on the standard curve, corresponded to the activity of about 1 ng/cm^2 of peroxidase in solution. These coverslips were used to mimic the areas of the patterns that did not stain with diaminobenzidine in the experiments reported above. The figure is roughly 1% of the above figure, and is very satisfactory with regard to the aim of patterning a protein.

The other set of coverslips that were assayed in this way were those which had been ultrasonicated in acetone after peroxidase had been immobilised over them. This was tried because the procedure used to pattern BSA in the second method (so that it acts as a non-adsorbent surface) would be a good protein patterning method in itself, if only the protein stays in its native conformation after being treated with acetone. But this experiment shows that only about 10% of the activity is retained after such a treatment, and this is in the case of peroxidase, which is a fairly robust protein. For many applications, a loss of as much as 90% of the potential activity would be inadmissible, especially if 100% can be retained.

All in all, these results show that a good patterning method has been developed, one that is potentially versatile, that manages to treat the proteins gently enough so that they retain maximum activity, and that provides a very sharp distinction between the different areas that are created.

OTHER PROTEIN PATTERNING METHODS

There are other methods for patterning proteins described in the literature. The next paragraphs will give an outline of those methods and will compare them with the one that has been developed in this work. All these methods can be loosely classed into two different groups, one in which the patterning is achieved by selectively eliminating areas of a continuous layer of proteins, and another in which it is achieved by creating first a pattern of adherent and non adherent areas and then adding proteins.

A method that can be ascribed to the first group is one described by Faissner and co-workers, in a paper dealing with repulsive substrates for nerve cells (Faissner and Kruse, 1990). In brief, their method consists in adsorbing a continuous layer of protein (J1/tenascin) over a glass coverslip and scraping out lanes in it with the tip of a Eppendorf pipette. This method is clearly too crude to allow the production of patterns of any complexity.

Hammarback et al. described another method that can also be included in the first group, which they used to pattern laminin and fibronectin over glass slides to culture nerve cells on them (Hammarback, et al., 1985, Hammarback, et al., 1988). In short, they adsorbed the protein onto glass slides and then dried them. The dry slides were subsequently exposed to ultraviolet light partly protected by an electron microscopy grid, so that squares of protein were inactivated by the radiation (the mechanism of this inactivation is unknown), but lanes of it remained untouched. These patterns were successful in guiding the outgrowth of neurites. A paper by Peter Fromherz (Fromherz, et al., 1991)

takes this method a step further. In an attempt to guide neurite outgrowth in cultured leech nerve cells, he adsorbed leech laminin mixed with fluorescently labelled BSA in a continuous layer over glass coverslips. He then dried the coverslips and irradiated them through an electron microscopy grid. In this way, he was able to readily visualise the pattern by observing the unbleached fluorescence. Again, the method successfully produced patterns that guided neurite outgrowth. Yet another paper describes an attempt to use this method, in this case with less success (Bhatia, et al., 1991). In that work, the authors attempted to pattern antibodies, in a search for a general method for patterning proteins. They covalently attached antibodies labelled with radioiodine to a glass slide, and then irradiated the slides with deep ultraviolet light. They found that afterwards the radioactivity was well patterned, but when the activity of the antibody in the unirradiated areas was assayed, they did not obtain any consistent results. This highlights the fact that not necessarily all proteins will retain their activity after being adsorbed or immobilised and then dried, and so lacks the versatility of the method described in this thesis. In addition, the patterns that can be obtained are limited to the geometries of electron microscopy grids and similar objects, which are not very varied.

The next few works to be described deal with patterning methods that can be classed in the second group, that is, methods that rely on the production of a pattern of adherent and non adherent areas prior to the addition of the protein.

Cooper et al. describe a method for patterning growth of neuroblastoma cells that is based on protein patterns (Cooper, et

al., 1976). They deposited silicon monoxide through a slot onto a tissue culture plastic dish to produce lines about 4 μm wide, and then incubated the dishes with serum. Neuroblastoma cells cultured on that surface only grew along the lines, which suggests that the proteins in serum that promote neurite outgrowth from neuroblastoma cells, adsorb either preferentially or in a more native configuration to the silicon monoxide lines. The paper does not investigate or discuss that possibility, or indeed any reason for the cells preference of substrate. In any case, this method would not allow the construction of sophisticated patterns, and is quite limited in the choice of surfaces over which the patterns can be made.

Hammarback and Letourneau describe two methods that can be classed in the second group, but which bear close resemblance to their method discussed above. The first one (Letourneau, 1975) relies on shadowing palladium vapour onto tissue culture plastic dishes protected by an electron microscopy grid, and adsorbing proteins (collagen or polyornithine) onto the surface afterwards. This was again used to culture nerve cells, and indeed the cells grew preferentially on the lanes where no palladium had been deposited, a fact that points out that either proteins were not being adsorbed to the palladium or, if they were, only in an inactive conformation. In another paper (Hammarback and Letourneau, 1986) they adsorbed a mixture of agarose and BSA onto the slide, irradiated it through an electron microscopy grid and then added laminin, which was adsorbed only onto the irradiated areas. Yet again, they cultured nerve cells over these surfaces, and neurites only grew on the irradiated areas where the BSA/agarose had been eliminated and

replaced with laminin. The shortcoming of these two methods, when considering them as general ways of patterning proteins, is that it is not possible to covalently bind the patterned proteins to the substrate, and many proteins loose a high percentage of their activity when simply adsorbed. In any case, all the methods described by Hammarback and co-workers could be greatly improved by substituting the electron microscope grids for chromium masks of the type used in photolithography; one problem might be that these are going to make very tight contact with all the surface where the protein is, so that the pattern could be damaged.

Vielmetter et al. developed a method that relied on physically blocking the adsorption of the proteins (derived from the ECM and cell surfaces of the nervous system of goldfish) to the substrate (Vielmetter, et al., 1990). To do that, they made a silicon matrix with grooves 50 μm wide separated by bars 40 μm wide, in such a manner that, on both ends, the parallel grooves merged into an inlet and an outlet channel, respectively. They then placed it on top of a glass surface and allowed the protein solution to diffuse through the inlet channel, so that it would only come into contact with the glass under the grooves. After this they washed the grooves through the inlet channel, and finally they removed the silicon matrix from the glass. The purpose of this was again to culture cells on the glass surface, and the cells clearly sensed the patterns and grew aligned to them. The problem with this method is its resolution; It would be very difficult to manage geometries with a scale any smaller than the one described.

Bhatia and co-workers (Bhatia, et al., 1993) discuss another method for patterning proteins. In it, they laid a layer of mercaptopropyltrimethoxysilane (MTS) over a glass surface, and irradiated it with deep ultraviolet light through a mask. The irradiated areas reportedly lost the sulphydryl reactivity, and moreover became resistant to protein adsorption, so the proteins could then be either cross-linked or adsorbed onto the unirradiated areas. They tried this method with BSA and with antibodies, in a work exclusively oriented towards the development of a protein patterning technique. The results seemed to vary from protein to protein. When using antibodies, the adsorbed activity was reduced by 86% if the slide was irradiated, but when using BSA, the amount adsorbed was only reduced by 74% by the irradiation. A remnant of 26% of the protein, when none should remain, would be quite unacceptable in many cases, and so probably this method would have to be reserved for only those proteins whose adsorption to irradiated MTS is minimal.

Another method, developed by Nakamoto and co-workers (Nakamoto, et al., 1988), is described in a paper dealing with the preparation of enzyme-immobilised membranes of multibiosensors. In this case they attempted to couple several different enzymes on the same membrane, with micrometer geometries. To do this, they followed a procedure that bears some resemblance to that developed in the course of this thesis, and which relies on the technique of photolithography. They would first cover a slide with photoresist, then expose it to ultraviolet light, develop it, then couple an enzyme to the area bare of resist, and finally wash away the remaining resist with acetone. After

this, they would cover the slide in photoresist again, immobilised enzyme and all, and repeat all the steps to obtain a slide with two different well defined regions covered with two different proteins. The process would be repeated as many times as needed, with as many different proteins as wanted. The authors of the paper reported good results, and good conservation of enzymatic activity. However, in the course of the work leading to the present thesis, it was found that acetone washings of an immobilised enzyme, horseradish peroxidase, rendered it inactive by as much as 90%, as described in the section dealing with peroxidase assays. Certainly, this method would be the ultimate protein patterning technique if greater activity could be salvaged from the resist stripping step. In any case, it could be useful when more than one protein has to be laid on a pattern and the retention of activity is not vital.

Next, the work carried out by people in the same group as the author of this thesis will be discussed. Two papers (Britland, et al., 1992, Clark, et al., 1992) describe cells cultured over patterns of chlorosilane and aminosilane, made approximately with the same procedure as that described in the Methods section. These cells clearly are influenced by the patterns, and their growth is oriented by them, preferring aminosilanated to chlorosilanated areas. The patterns were not incubated with any protein before adding the cells; but 10% of the culture medium was serum, so serum proteins were most probably adsorbed to the pattern. As has already been discussed, chlorosilanated glass is probably not a non-adsorbing surface for proteins, so an explanation for the behaviour of the cells could be that the adhesive serum proteins that adsorbed to the chlorosilane did so in an inactive form. This

possibility is hinted at in the paper by Britland et al., and patterns preincubated with whole serum were tested. These patterns were much less effective in guiding the adhesion and growth of the cells; so, clearly, another explanation is needed. It could be that BSA was adsorbed to the chlorosilane, after the medium and the cells were added, whereas adhesion proteins were adsorbed to the aminosilane. BSA is by far the most abundant protein in serum, so it would be the first to come into contact with both surfaces and so to get adsorbed to them. However, its binding to chlorosilane would be stronger than its binding to aminosilane, so the bigger, more adherent cell-adhesion proteins (fibronectin, vitronectin, thrombospondin) would take much less time in displacing BSA from aminosilane. This agrees with the fact that incubation of the patterns with whole serum diminishes its ability to orientate cells: more cell-adhesion proteins over a longer period were able to displace the majority of the BSA adsorbed to chlorosilane.

A following paper (Britland, et al., 1992), in which the author of this thesis was involved, dealt with using the method described in the preceding paragraph to develop a general method to pattern proteins. This is the first of the two protein patterning methods described in this thesis, and has been discussed at length earlier on, with the conclusion that it was not completely successful, although results at the time of the submission of the paper seemed to indicate the opposite. In the same paper another development is described, which consisted on growing peptides with the method of Merrifield in the aminosilanated areas of the patterns. This proved to be feasible,

though of course only fairly short peptides could be grown in this way.

Finally, a paper by Clark (Clark, et al., 1993) will be briefly discussed. In it, nerve cells were cultured over patterns of chlorosilane made over bare quartz and preincubated with the protein laminin. Neurites from the nerve cells did grow on, and were oriented, by the pattern, and this time they seemed to prefer the protein adsorbed to the chlorosilanated areas. Immunofluorescence localisation of laminin showed that it was preferentially accumulated on the hydrophobic parts of the pattern, a fact that is in agreement with the nature of protein adsorption discussed above. The fact that the neurites avoided the proteins adsorbed to quartz can be explained in two different ways. One is that laminin was being displaced by the more abundant proteins in the culture medium; the other is that laminin is adsorbed to quartz in a way that does not encourage neurite outgrowth. The fact that laminin seemed to be accumulated over the chlorosilane suggests that the first explanation is more likely. Or a combination of the two: there are reports that neurite outgrowth does not occur over laminin adsorbed onto plain glass, even in the absence of serum in the medium (Rogers, et al., 1983).

All in all, I think that it can safely be concluded that the method developed in the course of this thesis is the most versatile and reliable of all the described methods. Most of these have been developed for a particular purpose and give good performances when applied with the protein for which it was intended, but would most probably fail if applied to other proteins. On the other hand, the methods that would produce

patterns with any protein lack geometric versatility. The method presented here has all the geometric versatility of photolithography, which is in fact the maximum any other method achieves. At the same time, it ought to be possible to pattern virtually any protein with it, because if for some reason BSA is adherent to a specific protein, there will always be thousands other proteins which are not, and which can replace BSA without any major change in the procedure.

SECOND PART:

NEURITE OUTGROWTH GUIDANCE

INTRODUCTION

This second part discusses the fabrication of patterns of the protein laminin, designed to maintain cultures of nerve cells with the aim of ordering and directing the growth of the axon-like processes (neurites) sprouting from the cell bodies. In this way, it should be possible in the long term to construct nerve cell circuits *in vitro*. The investigation of such circuits should be of interest in areas such as the study of the behaviour of growing dendrites and axons, the study of models of neural circuits that exist in living nervous systems, or the development of neural network computing.

This introduction will start by examining the mechanisms of neurite outgrowth, and then laminin, the relation between the two and will end by outlining the rationale behind the experiments to be described.

NEURITE OUTGROWTH

Neurites are the axon-like processes that sprout from the body of nerve cells when these are placed *in vitro* under appropriate conditions. They resemble developing axons and dendrites, and are a good model to study some of their characteristics (indeed, when axons and dendrites are growing during development *in vivo* and are indistinguishable from each other, they are also called neurites). They consist of a long and thin tubular section, often ramified, and a flattened spread end, the growth cone. It was Ramon y Cajal (Ramon y Cajal, 1890) who first described growth cones and suggested their role as the motile elements in neurites, after painstakingly studying a considerable number of fixed sections of embryonic retinas. Ross Harrison (Harrison, 1910) took the next step, by culturing

fragments of developing amphibian nerve tissue in drops of clotted lymph, and in that way he was able to observe and describe for the first time growing live neurites.

After their work, many researchers have devoted their time to unravelling the mechanisms by which neurites grow and by which they choose the direction towards which they extend. To a large extent, it is the control of these that determines the vastly complex pattern of neuronal interconnections in the brain. (For a review of the work of the leading researchers in that area up to the 60's, see the addendum at the closing of this thesis)

NEURITE STRUCTURE

Neurites consist of a tubular section, sometimes called the shaft, and at the distal tip of the shaft, a wider, irregularly shaped section, called the growth cone (see figs. 2.1 and 2.2). The shaft is the part that remains in place after the neurite has reached maturity, and it becomes functional as an axon or a dendrite. It can be of any length, up to about one meter in adult human beings (and much longer in animals such as whales), and its diameter varies from somewhat less than 1 μm to about 1 mm in giant axons of squids. This latter figure is very uncommon, though, and the large majority of nerve fibres are no more than a few μm in diameter.

A plasma membrane, sometimes called the axolemma, surrounds the neurite all along its length, and governs the exchanges between the interior and the extracellular medium. The interior of this membrane is lined by a thin cortical layer of actin filaments (Bunge, 1973) that may keep it rigid. The core of the shaft is occupied by parallel bundles of microtubules with an

average length of 100 μm each, always with their plus ends (where polymerisation of tubulin subunits occur) located distally (Ahmad, et al., 1993). These microtubules have frequent cross-bridges that integrate them into an extensive network. The periphery of the zone occupied by microtubules is filled in addition by neurofilaments, also oriented parallel to the shaft axis, and with cross-bridges between them and with the microtubules (Okabe, et al., 1993). Some cellular organelles can be detected associated with the microtubules, especially mitochondria, small smooth vesicles and some smooth endoplasmic reticulum. These organelles are transported along the distance of the neurite in association with the microtubules, in what constitutes the fast axonal transport.

The growth cone is seen as a widening of the tip of the shaft of variable area, with a rather ragged outline dominated by filopodia and lamellipodia. Filopodia are rod-like processes on the edges of the growth cone, a fraction of a micrometer in diameter and up to several tens of μm in length. They extend from the leading edge, swing erratically from side to side, apparently testing the environments of the growth cone, and retract within a few minutes of extending (Bunge, 1973, Yamada and Wessells, 1971). Lamellipodia are flat, thin, membranous structures, that also expand and retract from the advancing growth cone, although not so actively, and display a continuous centripetal ruffling in the membrane. The relative amount of filopodia and lamellipodia varies considerably depending on the cell type and the conditions surrounding the neurite (Bovolenta and Mason, 1987).

At the point at which the shaft begins to widen into the growth cone, the microtubules cease to be arranged in a bundle and adopt a variety of conformations, and they occupy the majority of the intracellular space of the growth cone. They are almost never seen to penetrate into the lamellipodia, though, and never into the filopodia (Sabry, et al., 1991). That space is occupied almost exclusively by actin filaments, that form either tight bundles of parallel filaments in the filopodia, or a fine meshwork in the lamellipodia. The bundles are approximately 0.2-0.3 μm wide and 10 μm long, much shorter than typical actin fibres in fibroblasts or epithelial cells (Letourneau, 1981). The actin filaments in the filopodia have their barbed ends (where polymerisation occurs) oriented towards the tips of the filopodia, and the filaments in the lamellipodia have in general an orientation consistent with that of the filaments in the bundles. Myosin is also present in the growth cone, mainly in the lamellipodia and in the base of the filopodia, and to a lesser extent inside the filopodia (Letourneau, 1981, Bridgman and Dailey, 1989). The area of the growth cone dominated by the presence of microtubules is sometimes called the C-domain (from Centre), whereas the area dominated by microfilaments is called the P-domain (from Periphery). Both domains are rich in actin and tubulin monomers. In addition, the C-domain has numerous organelles that are not present in the P-domain. These include mainly a specialised form of smooth endoplasmic reticulum with a reduced lumen, that probably acts as a calcium storage organelle (Dailey and Bridgman, 1991), mitochondria, and small smooth vesicles. Among these vesicles, there are synaptic vesicles, and a large pool of vesicles with a diameter around 180

nm, that provide the membrane necessary for neurite extension, and a lesser amount of smaller, vacuole-like structures (Lockerbie, et al., 1991).

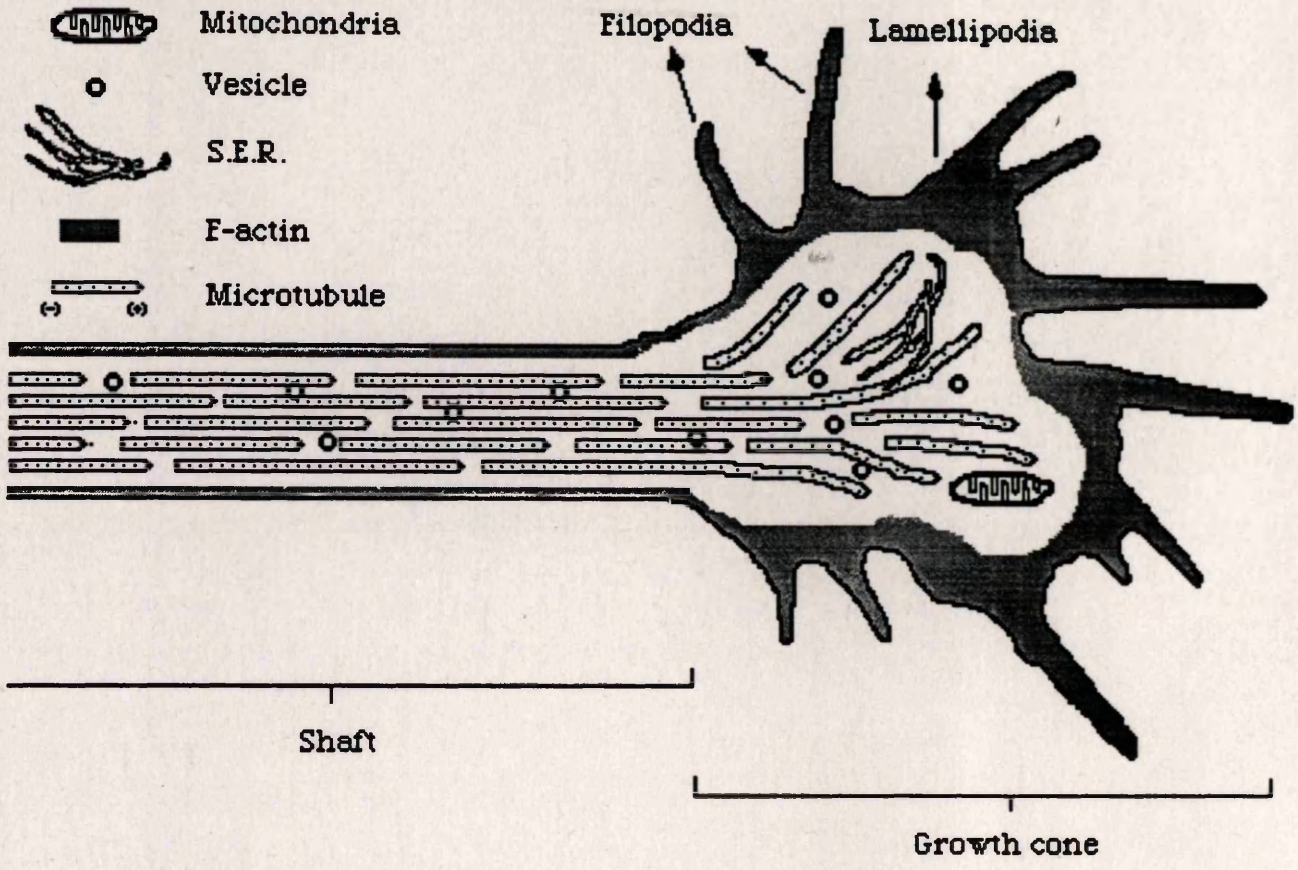


Fig. 2.1: Schematic diagram of the terminal region of a neurite.

DYNAMICS OF CYTOSKELETAL COMPONENTS IN THE GROWTH CONE

It has been known for quite a long time that both the microfilament and the microtubule systems are essential for the outgrowth of neurites. This was first shown by Yamada et al. (Yamada, et al., 1970), who described the effects of cytochalasin B and of colchicine on dorsal root ganglion cells from chick embryos cultured on tissue culture plastic. They found that cytochalasin B,

Fig 2.2: Nomarski optics micrograph of the growth cone of an outgrowing neurite from a DRG cell, cultured over laminin adsorbed to aminosilanated glass. Bar: 10 μm.

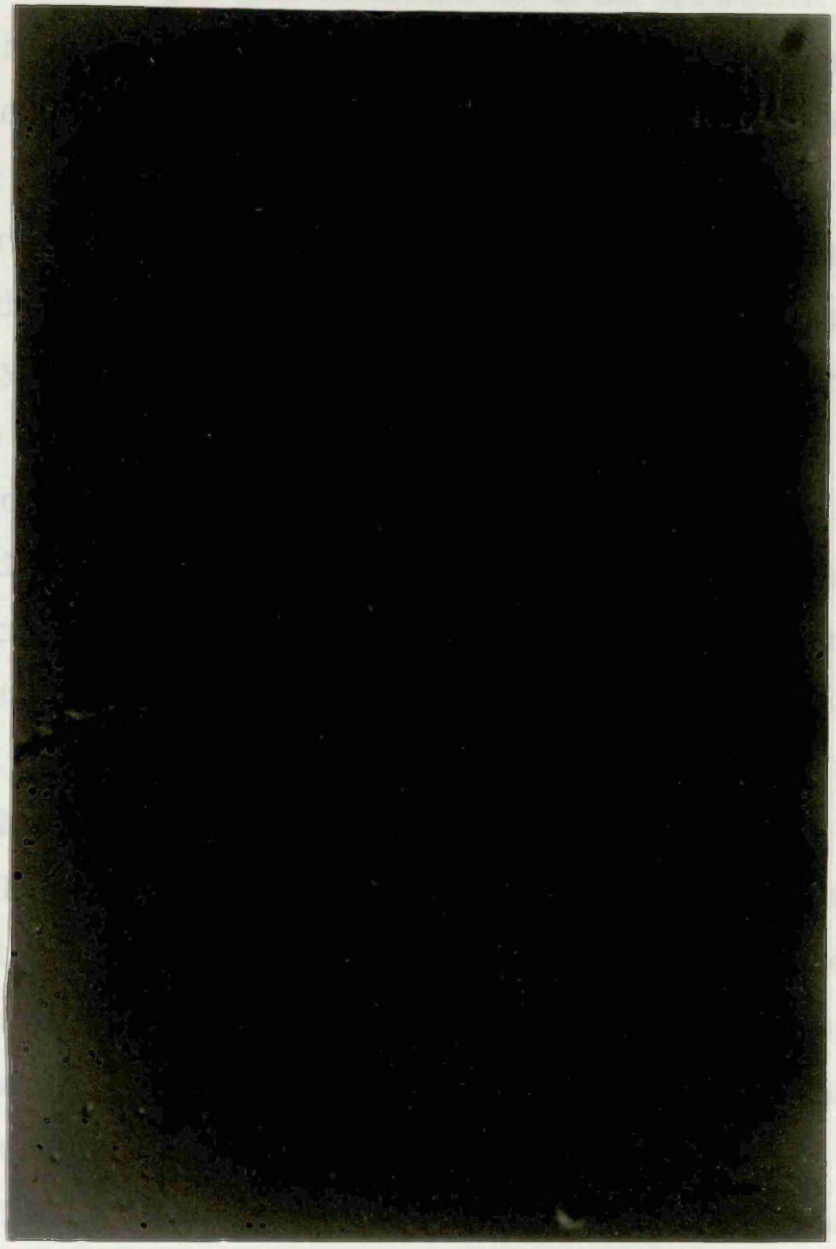


Fig. 2.2

which disrupts the structure of actin filaments, caused the collapse of the growth cones and the cessation of neurite extension. On the other hand, colchicine, a drug which produces the disassembly of microtubules, caused shortening and retraction of neurites, but without initially affecting the structure of the growth cones. Neurofilaments, in their turn, do not seem to play any mayor role in the extension of neurites, and appear in the neurite only when a section is well established (Okabe and Hirokawa, 1992). Thus, in order to understand the mechanisms underlying neurite outgrowth, it is important to know how the microtubule and the microfilament systems are assembled in the neurite and how they evolve while the neurite is growing.

Microtubule dynamics

There have been two main schools of thought as to the origin of the microtubules that occupy the interior of neurites. One argues that they are mainly assembled in the cell body and are then translocated into the neurites, whereas the other maintains that they are assembled anew inside the neurites.

There are a number of papers supporting the idea that microtubules are nucleated in the cell body and then transported into the neurite. One piece of evidence for this is that γ-tubulin, a protein that plays a critical role in microtubule nucleation, can be detected in the cell body of nerve cells, but not in the growing neurites (Baas and Joshi, 1992). Another piece of work that supports this idea was described in a paper by Reinsch et al. (Reinsch, et al., 1991). Observing microtubules labelled with a fluorescent marker, they found that these move consistently away from the cell body all along the shaft of the neurite,

whether the neurite was extending or not. This advance was faster as the distance from the cell body increased, until near the growth cone it nearly matched the speed at which the neurite was growing. Furthermore, the speed at which the microtubules advanced very near the growth cone was not constant, but seemed to reflect the fluctuating behaviour of the growth cone. The acceleration of the movement of the microtubules along the neurite would be balanced by polymerisation of tubulin into the microtubules while they are in the neurite. Tanaka et al. (Tanaka and Kirschner, 1991) and Ahmad et al. (Ahmad, et al., 1993) observed that microtubules are longer the farther away they are from the cell body, and they took it as evidence that polymerisation occurs.

On the other hand, there is also experimental evidence that suggests that microtubules are mainly incorporated into the neurite by net assembly at the tip. Lim et al. (Lim, et al., 1990) concluded this when they looked at bleached zones in fluorescently labelled microtubules inside growing neurites, and saw that they were stationary. Edson et al. (Edson, et al., 1993) also observed that microtubules were much more stable in regions of the neurite proximal to the cell body than in distal regions. Another piece of evidence in this respect comes in a paper by Zheng et al. (Zheng, et al., 1993). These authors observed the growth of neurites in the presence of the drug vinblastine, which prevents net polymerisation of microtubules but does not depolymerise existing ones. The newly formed neurites (that were artificially started by applying tension on the cell body) had extremely short non-functional microtubules, that could not assemble into bundles. In contrast, the cytoplasm of the

cell body had an almost normal population. Finally, Okabe (Okabe and Hirokawa, 1992), looking at the incorporation of labelled tubulin into neurites, came to the conclusion that they are incorporated mainly as subunits and not as polymers.

In the end, it seems demonstrated that neuritic microtubules are nucleated in the cell body, and that they are very dynamic inside the neurite. On the other hand, it seems probable, as noted by Edson et al. (Edson, et al., 1993), that the two mechanisms discussed above cooperate during neurite extension, and depending on the conditions and on the cell type one or the other will make the bigger contribution.

It also seems clear that the microtubules near the growth cone are more dynamic than those in more proximal regions. This was noted by Edson et al. (Edson, et al., 1993), studying the recovery of bleached zones in neurites filled with fluorescent tubulin. Ahmad et al. (Ahmad, et al., 1993) came to the same conclusion when they looked at the level of tyrosination of α-tubulin with electron microscopy (a high level of tyrosination indicates high lability). The opinion of the latter was that the higher lability was indicative of the need of the microtubules to react to the fluctuations in the advance of the growth cones. In addition to this, the set of microtubules inside the growth cone adopt a variety of conformations that seem to match the speed and direction of growth cone advance (Tanaka and Kirschner, 1991). They seem to foreshadow the future behaviour of the growth cone, and to switch their conformation to fit that behaviour very rapidly. When the growth cone is about to advance faster, they adopt a bundled conformation, and this bundle points in the future direction of growth. During periods of slower advance,

they adopt a splayed or looped conformation, and in these cases they grow and shrink very rapidly. Sabry et al. (Sabry, et al., 1991) also observed that when grasshopper growth cones *in vivo* contact a guidepost cell, the microtubules selectively invade the branches created by the filopodia that had made the contact.

Microfilament dynamics

There seems to be no doubt about the fact that the incorporation of G-actin into microfilaments occurs at the distalmost, membrane-associated, barbed ends in the lamellipodia. Evidence for this can be found in a paper by Okabe and Hirokawa (Okabe and Hirokawa, 1991). These authors injected biotin labelled actin into nerve cells, and afterwards fixed them and looked for the biotin by EM. With short incubation periods, it was mainly concentrated in the outer borders of lamellipodia, and with longer incubations the biotinated area expanded inwards until all the microfilaments were marked with biotin.

There is also enough evidence to indicate that there is a continuous centripetal movement of the whole microfilament meshwork in the growth cone. For example, Forscher and Smith (Forscher and Smith, 1988) applied cytochalasin B (CB) to growth cones *in vitro* and found that, immediately after addition, the microfilament meshwork disappeared from the outer borders of the lamellipodia, and then this deletion extended inwards until there were no microfilaments left. Upon withdrawal of the CB from the medium, microfilaments appeared in the distal borders of the lamellipodia, and then spread centripetally. More evidence comes in the paper by Okabe and Hirokawa (Okabe and

Hirokawa, 1991). They injected nerve cells with fluorescein-labelled actin , and when all microfilaments were fluorescent, they photobleached small areas of the meshwork and looked at its evolution. They found that the bleached areas moved centripetally from the edges, and that this movement could not be accounted for by diffusion, as the radii of the bleached zones remained almost constant. Similarly, when they bleached filopodia, recovery of fluorescence started at the tips and then proceeded towards the base.

There seems to be an agreement that when the filamentous actin reaches the C-domain, it depolymerises, and the resulting G-actin diffuses in the growth cone to be incorporated again in the leading edge into microfilaments. There is no direct experimental evidence for this, but if it were not so, the central area of the growth cones would be crammed with microfilaments, and it is not.

The motor behind the centripetal flow of actin in the growth cone could be myosin, and a number of authors support this idea, although the only evidence for it is the co-localisation of actin and myosin mentioned above.

PUSHED VERSUS PULLING GROWTH CONES

Ramon y Cajal was the first to suggest that the part of the neurite that powers its extension is the growth cone, and Ross Harrison agreed with him on this respect (Ramon y Cajal, 1890, Harrison, 1910). More recently, this idea was expanded with the concept that growth cone advance is due to the exertion of traction force by filopodia anchored to the substratum. The force would be produced by a muscle like, actin-myosin mechanism.

The basis for this assumption was not so much direct evidence as repeated observations of myosin and actin localised together in the growth cone (Letourneau, 1981, Bridgman and Dailey, 1989).

Letorneau (Letourneau, 1975, Letourneau, 1975) studied the behaviour of neurites when presented with a choice of substrates, and observed that they would always extend along the substrate of maximum adhesivity. Thus, he proposed that filopodia would adhere to the different substrates with varying strength, and would compete with each other to pull the neurite in their direction, in what he described as a sort of "tug of war". The filopodia that had adhered with maximum strength would in the end provoke the others to detach and the neurite would thus follow only those.

This model was challenged by subsequent observations. For example, it was noted (Marsh and Letourneau, 1984) that nerve cells cultured on highly adhesive substrate in the presence of cytochalasin B were able to extend neurites, although fairly abnormal ones. Cytochalasin B disorganises actin filaments and produces growth cones lacking in filopodia and lamellipodia, so some mechanism independent of those structures had to be responsible for neurite extension in this case. Also, some researchers (Goldberg and Burmeister, 1986, Aletta and Greene, 1988) noted that fast advancing growth cones were rich in cytoplasmic protrusions and had sparse or no filopodia, whereas growth cones advancing slower or not advancing at all were very rich in filopodia. These observations led to the hypothesis that the advance of growth cones was due to microtubule-rich cytoplasm pushing forwards, and that the role of filopodia was only that of choosing the direction of movement.

Again, new observations reversed the opinion, and now there is some consensus on the idea of growth cones pulling on the neurites. One such observation was made by Heidemann and Buxbaum (Heidemann and Buxbaum, 1990). They made direct measurements of the tension produced by growth cones and saw that it correlated with the speed at which they were advancing. Another such observation was that growth cones were capable of dragging behind the cell bodies when these were detached from the substratum, without neurite elongation. Similarly, growth cones severed from the cell body would keep on crawling for a while (Bray, 1987).

Heidemann and his co-workers studied the production of traction force by growth cones by observing them under conditions that translated the traction force to the displacement of obstacles in the environment, such as neurites and glass fibres (Heidemann, et al., 1991). They found that the displacement of these obstacles occurred mainly through filopodial contractions. They thus concluded that growth cones produce the traction force needed for their advance through the contractile activity of filopodia. Nevertheless, in a subsequent paper, these authors argued against the "tug of war" steering mechanism (Lamoureux, et al., 1992). They found that applying to a growth cone or a filopodium 5-10 times the tension it itself is able to generate did not detach it from even the least adhesive of the substrata. They thus concluded that another mechanism is needed to explain the navigational capabilities of growth cones. Models for this will be introduced in a later section of this introduction.

GENERATION OF TRACTION FORCE BY GROWTH CONES

There is a consensus on the idea that traction force is generated in the growth cones by the interplay of actin, myosin and adhesion points. Quite how these three elements are arranged and how they interplay to produce the force is at the moment a subject of speculation.

Smith (Smith, 1988) argues that the forces driving growth cone motility are fundamentally ATP-energised actin polymerisation and actin-myosin interactions. Actin polymerisation would propel the protrusion of lamellipodia and filopodia, whereas the interaction between actin and myosin would produce traction force. If no adhesion is established by the protrusion, traction force would provoke its retraction and reabsorption. If adhesion is established, traction force would produce tension on the rest of the growth cone.

Heidemann et al. (Heidemann and Buxbaum, 1990, Heidemann, et al., 1991) propose a somewhat different picture. In their model, it is only tension generated by filopodia that produces growth cone advance. If a filopodium adheres to the substratum, myosin pulling on the part of the actin bundle that is inserted on the leading edge would create tension. If this tension is greater than a given threshold, the thixotropic quality of the actin meshwork in the leading edge would provoke a transition from a gel state to a sol state, and the consequent relaxation of that part of the cytoplasm would cause an engorgement of that part of the leading edge. The resulting cessation of the exerted tension would now cause a reversion to a gel state, thus consolidating the advance. In this case, only the advance of the filopodia would be powered by actin polymerisation.

For the mechanism of filopodium dynamics, Sheetz et al (Sheetz, et al., 1992) advance a different model. The actors are the same, but arranged in a slightly different manner. The main addition of this model is that the protrusion is not powered by actin polymerisation but by myosin interaction with actin. Myosin anchored in the membrane of the tip of an unadhered filopodium would pull on the actin bundle. But with the bundle well anchored back in the actin meshwork in the leading edge, the only effect of this pulling would be to thrust the membrane forward, thus providing space for more addition of G-actin into the bundle. The retraction of the filopodium would occur when this filopodial myosin stopped pulling and myosin at the base of the filopodium started pulling instead.

Whatever the exact mechanism, it seems that it is largely filopodial activity that propels growth cones forward.

INTEGRATION OF GROWTH CONE ADVANCE WITH NEURITE EXTENSION

Without neurite extension, the activity of the growth cone would only amount to protrusions and retractions with no net displacement. Neurite extension is accomplished by the elongation of the microtubule bundle in the direction of growth (Tanaka and Kirschner, 1991, Goldberg and Burmeister, 1989, Gordonweeks, 1989). Related to this topic is a fact that would explain the observation by Marsh et al. (Marsh and Letourneau, 1984) to the effect that CB treated nerve cells on highly adhesive substrates produce neurites. This is the presence in nerve cells of a microtubule binding protein (MAP2c) that stabilises and promotes microtubule bundling (Weisshaar, et al., 1992). Non-

neuronal cells transfected with the MAP2c gene and treated with CB produce processes reminiscent of those described by Marsh et al. So the contribution of microtubule bundling on neurite extension seems clear.

The mechanisms that need clarification are the ways by which the environmental signals each filopodium encounters are integrated so as to choose which of them leads the way, and how these signals direct microtubule bundling. One thing that is established, though, is that filopodia have receptor molecules in their membranes, sometimes associated with ion channels, and are capable of receiving external signals and of transducing them (Davenport, et al., 1993). One advantage of using filopodia as sensing organs would be that, due to their very large surface area to volume ratio, small changes in membrane permeability lead to enormous changes in the intrafilopodial chemical composition, making them extremely sensitive to weak signals. Sabry et al. (Sabry, et al., 1991) propose that filopodia encountering appropriate signals create in their base a concentration of second messengers that promote the invasion of microtubules, and that this eventually provokes the prolongation of the microtubule bundle in that direction.

Buxbaum and Heidemann (Buxbaum and Heidemann, 1992) advance a model in which tension acts as the integrating factor between signals received by filopodia and the extension of the microtubule bundle. They propose that somehow these signals produce an increase in the tension exerted by the filopodia upon the actin meshwork at its base. This causes the solification of that meshwork, as explained earlier, and as it was the gel state of actin that stopped microtubules from elongating, these will

extend towards the area suddenly devoid of physical containment, thus extending the whole neurite in that direction.

The addition of new membrane necessary for neurite extension is accomplished in the growth cone. The source of membrane is a set of Golgi-derived vesicles associated with the microtubules, which are transported to the growth cone by fast axonal transport, and their fusion with the axolemma seems to be a regulated process (Lockerbie, et al., 1991). Fast axonal transport is essential for neurite elongation, but not for growth cone motility (Martenson, et al., 1993) These authors conclude that fast axonal transport is probably responsible for the supply of cytoskeletal components necessary for neurite elongation; however, it could be equally possible that it is the supply of membrane components that makes fast axonal transport essential.

MODULATORS OF GROWTH CONE MOTILITY

Many modulators of growth cone motility have been described, both physical and chemical. They are factors that when encountered by an advancing growth cone, influence its rate of movement or its direction of advance. These factors are generated both by the target cells and by cells in the path towards the target. The receptors for most of these modulators are placed in the membrane of the growth cone (Reichardt, et al., 1992, Strittmatter and Fishman, 1991), and they presumably interact with the cytoskeleton through a variety of second messenger systems (Bixby and Harris, 1991, Lankford and Letourneau, 1991). Growth cones from different nerve cell types

may respond to different sets of modulators, or indeed respond in different ways to the same modulator.

Many molecules from the extracellular matrix (ECM) act as modulators of growth cone motility. Among these are laminin, merosin, fibronectin, vitronectin, thrombospondin, collagens, proteoglycans, and variants of all these, with more being described every year. These molecules sometimes act as a permissive substratum, stimulating the motility of growth cones, and for example laminin and fibronectin act as such for sympathetic neurones from chick embryo *in vitro* (Rogers, et al., 1983). It was thought that this stimulation of motility was due to the adhesiveness of these proteins for the growth cones (Hammarback, et al., 1988), but now it seem clear that they produce their effect by the mediation of receptors and probably second messenger systems (Calof and Lander, 1991, Lemmon, et al., 1992). ECM molecules can also act as repulsive substrates, inhibiting growth cone motility, as does, for example, tenascin on murine CNS nerve cells (Faissner and Kruse, 1990), or chondroitin sulphate proteoglycans on PC12D cells (Oohira, et al., 1991), both tested *in vitro*. There seems to be evidence that, at least in some cases, extracellular molecules are arranged in a path that the growth cones can follow to reach their destination *in vivo* (Riggott and Moody, 1987). Also, tenascin has been claimed to delineate the boundaries through which neurites do not penetrate *in vivo* (Steindler, et al., 1989).

Soluble molecules are also involved to some extent in affecting growth cone guidance. One classic example is nerve growth factor (NGF), which can act both as a chemotrophic (Levi-Montalcini, 1976) and a chemotactic factor (Campenot, 1977). Both

experiments with NGF were carried out *in vitro*, though, and the *in vivo* concentrations of NGF are probably not high enough to be chemotactic. Yet there is evidence for other substances acting as chemoattractants, that have not yet been identified (McCormack, et al., 1991).

Another factor that can influence growth cone behaviour is the set of proteins, and especially cell adhesion molecules (CAMs), present in the membrane of the cells that the growth cone encounters in its path. The number of CAMs that have been shown to be present in developing nervous systems is comparatively high, so that almost every CAM that has been described in other tissues seems to have a part to play here (and almost the same can be said of ECM molecules) (Hynes and Lander, 1992). The presence of certain CAMs in some neurites can lead to the growth cones following or diverging from other neurites, and thus to neurite bundling or fasciculation (Nagata and Nakatsuji, 1991). Growth cones in the developing limbs of grasshopper have also been shown to follow a path formed by specific non-adjacent cells, that have been called guidepost cells (Ho and Goodman, 1982). Glial cells play a part as well in modulating the motility of growth cones. Myelin, for example, has an inhibitory effect on most vertebrates, causing growth cone collapse. It is not yet known which of its components produces this effect, though (Bandtlow, et al., 1990).

Physical factors have also been reported to modify neurite outgrowth. One example is the effect of electric fields, that can, depending on the conditions, attract or deflect growing neurites (Patel, et al., 1985). Also, McCaig (McCaig, 1990, McCaig, 1990)

observed that a small electric field could induce branching of the neurites.

Another physical factor that affects the direction of neurite outgrowth is the topography of the solid support upon which the neurite elongates. Dow *et al.* (Dow *et al.*, 1987) studied the behaviour of nerve cells cultured over glass slides with micro-grooves and steps, and found that the neurites would tend to grow aligned parallel to these structures.

It is the combination of all these factors that achieves the establishment of all the connections that are necessary to produce a working nervous system. Not all of them necessarily act at the same time upon a given neurite. Typically, the pathway of a growth cone from cell body to target cell is made up of several stages, and each stage is managed in response to a set of factors that is specific for that growth cone and that stage. After the stage is over, the growth cone may change part of its receptors to respond to a new set of modulators (Hynes and Lander, 1992).

LAMININ

Among the extracellular matrix proteins that modulate growth cone motility, one of the better known is laminin. As laminin plays an important role in the work described in this thesis, it will be examined in some detail.

Laminin is a large glycoprotein of the extracellular matrix. It is encountered in significant quantities in the basal laminae, the thin extracellular matrix that surrounds epithelial tissues, nerves,

fat cells and muscles (Sanes, et al., 1990). It is abundant as well in the interstitial extracellular matrix of both the central and the

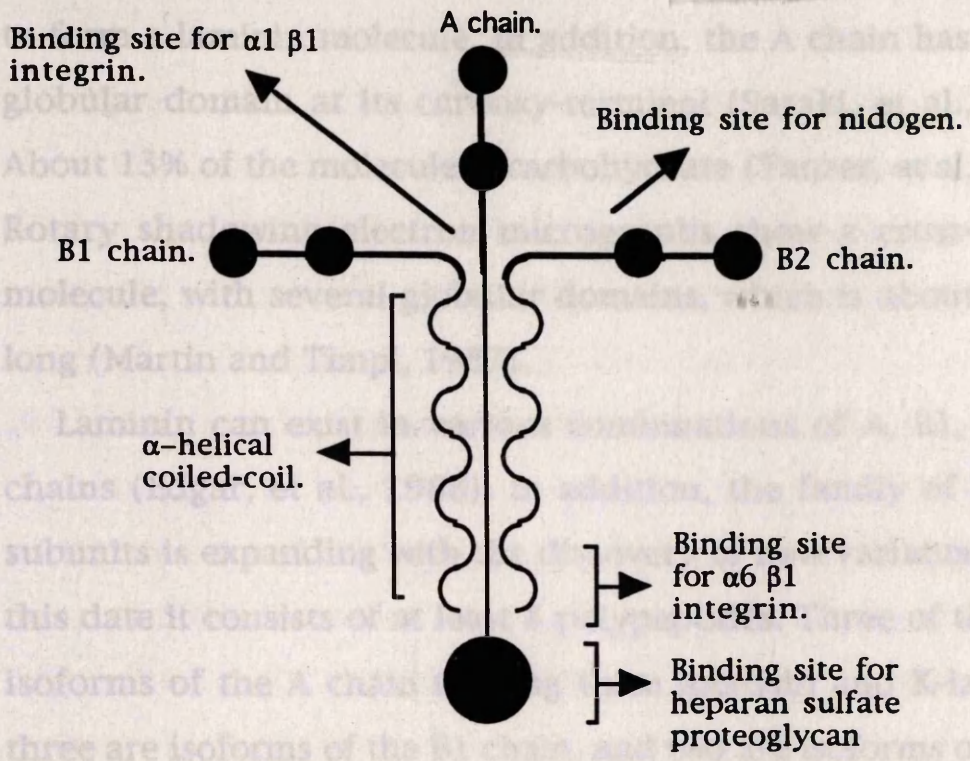


Fig. 2.3: Schematic diagram of a laminin molecule.

peripheral nervous system during development (Yip and Yip, 1992, Edgar, 1990).

Laminin was first isolated from a mouse tumour that produces large amounts of basal lamina, the Engelbreth-Holm-Swarm (EHS) tumour, and subsequently from human placenta. As isolated from these sources, it consists of three polypeptides, A, B1, and B2, with molecular masses (when unglycosylated) of, respectively, 400, 215, and 205 KDa (Timpl, et al., 1979). The genes for these polypeptides have been cloned, and their sequences indicate that the structures of the B1, the B2, and most of the A chain are similar. They consist, in their amino-terminal halves, of several

repeated EGF-like domains arranged sequentially, and in their carboxy-terminal halves, of a long α-helix. The α-helices of a B1, of a B2, and of an A chain get linked in a super helical coiled-coil to form a laminin molecule. In addition, the A chain has a large globular domain at its carboxy-terminal (Sasaki, et al., 1988). About 13% of the molecule is carbohydrate (Tanzer, et al., 1993). Rotary shadowing electron micrographs show a cross-shaped molecule, with several globular domains, which is about 70 nm long (Martin and Timpl, 1987).

Laminin can exist in various combinations of A, B1, and B2 chains (Edgar, et al., 1988). In addition, the family of laminin subunits is expanding with the discovery of new variants, and to this date it consists of at least 8 polypeptides. Three of them are isoforms of the A chain (among them merosin and K-laminin), three are isoforms of the B1 chain, and two are isoforms of the B2 chain (one of which is S-laminin). These polypeptides produce different combinations that are usually tissue specific (Decurtis and Reichardt, 1993, Marinkovich, et al., 1992).

Along its length, laminin has several attachment sites for other ECM components, notably collagen IV, proteoglycans, and nidogen (Martin and Timpl, 1987, Charonis, et al., 1985). It also self-assembles into polymers with a defined structure, in which each short arm inter-links with two others to form a hexagonal lattice (Yurchenco and Cheng, 1993). The presence of laminin is of great importance for the structure of basal laminae. Indeed, extracts from the EHS tumour matrix (which contain all the main components of basal laminae) can assemble, under physiological conditions, into a gel with a structure closely resembling that of

basal laminae; and this assembly is dependent upon the presence of laminin (Laurie, et al., 1986).

A large number of cell types have receptors for laminin, and several species of laminin receptors have been described. Some cell types express two or more kinds of laminin receptors at the same time, each of them eliciting a different response to laminin (Hall, et al., 1990). In different cell types, laminin can alter adhesion (Rogers, et al., 1983), survival (Edgar, 1990), morphology (Payne, et al., 1992), differentiation (Glukhova, et al., 1993), and motility (Liang and Crutcher, 1992).

Amongst the receptors for laminin, probably the best understood are those belonging to the major superfamily of ECM receptors, the integrins. Integrins are noncovalently associated heterodimeric glycoprotein complexes, composed of an α- and a β–chain. Each of the chains has a short cytoplasmic domain, a transmembrane domain, and a larger extracellular domain. At least 8 different β-chains have been described, and each of them can combine with one or more of the 14 α-chains that have been described to date, to produce a plethora of receptors. They recognise virtually all described ECM components (among others, laminin, fibronectin, collagens, vitronectin, tenascin, and thrombospondin), and also some cell surface molecules (VCAM-1, ICAM-1, ICAM-2). A given combination of an α– and a β-chain is not usually a specific receptor for a single ECM protein, but will often recognise several of them. At the same time, many of the molecules recognised by any particular integrin will often be recognised by some other members of the family. Different integrins may or may not share the same transduction pathway

when more than one of them are present in the same cell (Hynes and Lander, 1992).

Laminin is recognised by at least $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_7\beta_1$ integrins (Decurtis and Reichardt, 1993, Hall, et al., 1990, Glukhova, et al., 1993, Decurtis, 1993, Song, et al., 1992), although not all of them bind to the same part of the molecule.

One non-integrin laminin receptor that has claimed much attention is a 67 KDa protein. This was the first described laminin receptor, and was reported to bind to a specific sequence in the B1 chain. However, difficulties in pinning down its structure and characteristics, and doubts about it being a membrane protein, are making researchers lose interest in it (Mercurio and Shaw, 1991).

Other receptors that mediate laminin binding on cells are carbohydrate binding proteins. One of these is the galactose-specific lectin CBP 35. Antibodies against this protein are said to inhibit attachment of some cell types to laminin. Similar evidence has been obtained to the effect that a surface enzyme, galactosyltransferase, acts as well as a laminin receptor (Tanzer, et al., 1993). Finally, gangliosides and sulfatides present in the membrane of some cell types have been shown to bind to laminin (Reichardt, 1991).

EFFECT OF LAMININ ON NEURITE OUTGROWTH

The first to suggest that laminin might have a role to play during neural development were Rogers *et al* (Rogers, et al., 1983), when they showed that laminin adsorbed to tissue culture plastic promotes neurite extension on a number of nerve cell types. At the time, it was thought that laminin distribution was

restricted to basal laminae. Since then, it has been demonstrated that laminin is transiently present in complex patterns in the extracellular matrix of developing vertebrate nervous systems. For example, it is present in developing sensory and autonomic ganglia and spinal nerve roots (Rogers, et al., 1986), in the pathway of trigeminal nerve fibres (Riggott and Moody, 1987), in developing muscle prior to innervation (Sanes, et al., 1988), in the spinal ventral longitudinal pathway (Letourneau, et al., 1988), and in the optic nerve (Cohen, et al., 1986). In almost all cases the concentration of laminin is high during the period of axonal growth and then decreases to almost nothing. This is especially true in the central nervous system.

As demonstrated in the work mentioned earlier (Rogers, et al., 1983), laminin is certainly a substrate that allows and encourages neurite outgrowth from many nerve cell types. Nevertheless, there are many nerve cell types that do not respond in that way to laminin, and also cell types that do so only for a certain period of their development. The cells from the optic ganglia of chicken embryos, for example, are responsive to the EHS-type laminin only up to the time at which they reach their target, the optic tectum; then laminin stops having any effect on them, even though merosin is still capable of eliciting neurite outgrowth from them (Cohen, et al., 1986, Cohen, et al., 1987).

That laminin acts as a permissive substrate for neurite outgrowth *in vivo* as well as *in vitro* is supported by the fact that a monoclonal antibody, that binds to the neurite outgrowth promoting domain of laminin, stops axonal growth in regions of the peripheral nervous system in vivo (Sanes, et al., 1990). Also using laminin antibodies, Perris (Perris, et al., 1989) showed that

laminin is essential in pathways that direct migration of neural crest cells. What is not so clear is whether laminin plays a role in the guidance of neurite growth *in vivo* or it just allows growth, and other cues exist that guide developing neurites. There are a number of reports that demonstrate that tracts of laminin are capable of guiding neurite growth *in vitro* (some of them have been mentioned in the discussion in the first part of the thesis), but none that shows conclusively this fact *in vivo*. The work that comes nearest to showing this was reported by Riggot (Riggott and Moody, 1987). In it, it was shown that a punctuated pathway of laminin is present between the trigeminal nerve and the middle of the mandibular process just before innervation in chicken embryos. It is conceivable that, in absence of other cues, this pathway might direct the growing axons to their target. On the other hand, Yip and Yip (Yip and Yip, 1992) found that during the time at which ventral and dorsal root ganglia cells in chicken embryos send processes towards the neural tube and the dermamyotome, laminin immunostaining is present on the pathway, but not restricted to it; furthermore, antibodies against laminin injected prior to that time do not prevent the arrival of those axons at their target. Also, it is unlikely that laminin plays a role in neurite guidance in the central nervous system, because almost all nerve cells there are responsive to laminin and a huge number of neurites grow intermingled at the same time. It follows from all these facts that laminin probably acts as a permissive substrate, though not the only one, over which neurites navigate following a number of specific cues. In some cases, it can help to define the pathways of specific neurites, and

even be the main indicator of the pathway, specially in the peripheral nervous system.

The effect of laminin on neurite outgrowth is mediated largely by β1 integrins (Reichardt, 1991). Of all the β_1 integrins that can act as laminin receptors, $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ have so far been identified as mediating neurite outgrowth. $\alpha_1\beta_1$ and $\alpha_3\beta_1$, that recognise different neurite outgrowth promoting domains of laminin, have been shown to be expressed and active in the PC12 neuronal cell line (Tomaselli, et al., 1990, Tomaselli, et al., 1993). $\alpha_6\beta_1$ has been shown to mediate the laminin outgrowth promoting effect in chicken embryo retinal ganglion cells (Decurtis, 1993, Cohen, et al., 1987). Interestingly, in this last case, $\alpha_6\beta_1$ disappears when the cells stop being responsive to laminin; their responsiveness to merosin is mediated by another β_1 integrin.

There is evidence that suggests that the receptors for laminin, upon contact with the molecule, act on the growth cone apparatus through a mechanism which involves protein kinase C, at least in ciliary ganglion cells from chicken embryo (Bixby and Harris, 1991). This mechanism is at present very poorly understood, and much more research is needed in that direction. The final effect of the contact between a filopodium from a growth cone and laminin seems to be, first of all, that the filopodium gets strengthened and stabilised, and then it enlarges and fills with membranous organelles and microtubules, so that it becomes a established part of the neurite. If prior to this the neurite was not in contact with laminin, then the axonal transport and thus the rate of growth become faster.

All in all, we are still a long way from fully understanding the manner in which nerve cells interact with laminin (or with any

other extracellular protein) and what their real relationship *in vivo* is, but the foundations for a thorough understanding of the phenomenon seem to be laid.

NERVE CELL CULTURES.

As has been already pointed out, the first researcher to observe nerve cells *in vitro* was Ross Harrison (Harrison, 1910). He studied the outgrowth of neurites from frog and chicken pieces of neural tube cultured on drops of clotted lymph. After he abandoned this line of research, other researchers followed his lead, and their work up to the 50's is reviewed in the addendum. During the 50's there occurred a new impetus in cell culture in general, based on the development of growth mediums supplemented with antibiotics, and on improved sterile techniques. The culture of nerve cells, however, did not at that time advance as fast as the culture of other cell types. This was due to a basic difference between nerve cells and other cell types. Whereas most other cell types divide *in vitro*, and can thus be subcultured and cloned, and cell lines can be established, nerve cells do not divide *in vitro* (or *in vivo*, for that matter). For this reason, neurobiologists were forced to rely on heterogeneous primary cultures, in which glial cells abounded as much as nerve cells.

Two major breakthroughs in the field of *in vitro* neurobiology occurred in the 1970's. One was the development of neuroblastoma cell lines, obtained from neural tumours (Schubert, et al., 1969). These cell lines are able to proliferate in culture, can be subcultured and cloned. At the same time, they

can be induced to stop division and to acquire properties characteristic of differentiated nerve cells. The other breakthrough was the development of techniques to culture cells from autonomic and sensory ganglia, under conditions that offered new access to individual living nerve cells (Yamada, et al., 1970).

After that time, the field has kept growing, with countless improvements over old techniques. Perhaps one of the most important of these improvements was the development of a serum-free growth medium, by Bottenstein and Sato (Bottenstein and Sato, 1979). Also, the discovery of many ECM molecules and soluble factors (notably NGF) that promote nerve cell attachment and neurite outgrowth, as discussed above, has helped refine the techniques for nerve cell culture.

In this work, the cell type that has been used for experimentation was dorsal root ganglion (DRG) cells from chicken embryos. Chicken embryos are one of the sources of nerve cells most often used, probably because they are easily available and do not require much care. I decided to use primary cultures instead of a cell line, because it was assumed that the behaviour of neurites from freshly dissociated cells would reflect most the behaviour of neurites *in vivo*. Besides, there was no need, in our studies, for a genetically homogeneous source of cells. Nerve cells from the DRGs can be obtained easily with very little contamination of other cell types, and they produce very long neurites in a very short time, in just under a day, so they are ideal to study neurite behaviour.

Dorsal root ganglia are two series of sensory ganglia connected to the spinal cord, one on each side. They form during the first

2.5 days of incubation by a migration of cells from the neural crest. There are two different populations of sensory cells: One that is unresponsive to NGF, but responsive to brain-derived growth factor, and which degenerate in its absence. This population dies out when the ganglia are dissected from the spinal cord, for lack of this factor. The other, which is the one that survives in the cultures, is responsive to NGF. These cells differentiate between 8 and 15 days of incubation in the eggs. For this reason, the embryos were dissected during that period. Apart from nerve cells, DRGs contain some Schwann cells and fibroblasts. Most of these are eliminated from the initial cell suspension by pre-plating it over a surface with serum adsorbed onto it, as described in the methods section. The result is a fairly homogeneous population of nerve cells that produce long neurites within a day of culturing, if they are provide with NGF. After 48 hours, the non-neural cell that were not eliminated by preplating will start to be a major feature of the cultures, but by that time the most vigorous phase of neurite outgrowth will be over.

METHODS

PRODUCTION OF LAMININ PATTERNS TO ASSAY GROWTH CONE GUIDANCE

The procedure developed to pattern proteins has been used to produce patterns of laminin over which nerve cells have been cultured. The aim was first of all to see whether patterns of laminin, so produced, would be able to guide growth cones *in vitro*, and then to study neurite responses to different pattern geometries.

Laminin patterns were prepared by adsorbing laminin onto patterns of BSA made as described in the methods section of the first part. Basically, after completing step 10 as described for patterning proteins (step 9 can in this case be skipped, since modifying the amino groups of BSA is only required when the other protein is to be covalently coupled):

1) The coverslips were rinsed thoroughly in sterile PBS.

2) They were then incubated with 2 mls of a laminin solution (5µg/ml in sterile PBS) for 10 minutes at room temperature.

3) Then, rinsed thoroughly with sterile PBS.

4) Ultrasonicated in HEPES-buffered saline free of divalent cations (HS) for 1 minute.

5) Finally, the coverslips were placed in growth medium, and used on the day of preparation.

CELL CULTURE

The cells used in these studies were sensory neurones from the dorsal root ganglia (DRG) of chicken embryos, from embryonic day eight (E8) to E15. The procedure to obtain these cells is as follows (Lemmon, et al., 1992).

1) The chicken embryos were taken out of the eggs and placed in a petri dish filled with HEPES buffered saline free of divalent cations (HS).

2) The embryos were opened ventrally and gutted, and the DRGs were picked out and placed in HS.

3) The DRGs were transferred to 0.5% trypsin in HS, and incubated at 37°C for 10 minutes.

4) The DRGs were then transferred to 2% foetal calf serum medium (HEPES buffered Hams F-12 supplemented with 2 mM L-glutamine, 10 ng/ml NGF, 25 U streptomycin, 25 μg/ml penicillin, and Bottenstein N-2 mixture) and dissociated by gently pipetting them with a pasteur pipette 5 or 7 times.

5) The cells were plated in the same medium as above in a petri dish pre-coated with serum, and left at 37°C for one hour and a half. Whereas nerve cells do not adhere to serum coated plastic, fibroblasts and Schwann cells do, so this step eliminated most non-neural cells.

6) The non adherent cells were collected, counted, and plated on the chosen substrate with the same medium as above. Typically, 10,000 cells were plated per each 22x22 mm^2 coverslip, cultured for 24 hours at 37°C and then fixed with 4% formaldehyde in PBS (formal saline).

IMMUNOPEROXIDASE STAINING OF LAMININ SUBSTRATES

The laminin patterns had to be visualised after they had been used to culture nerve cells, so that it would be possible to see whether the newly produced neurites were following the laminin pathways. The method chosen for this was immunoperoxidase staining. This method is based on the use of antibodies coupled to

peroxidase. The pattern with the cells fixed with formal saline was first incubated with rabbit antibodies raised against laminin and then with goat antibodies raised against rabbit antibodies, the goat antibodies being coupled to peroxidase molecules. The end result of these incubations was that the peroxidase was precisely placed where there was laminin, and of course the position of peroxidase could be easily visualised with the diaminobenzidine assay described in the section dealing with peroxidase patterns. The details of the method are as follows:

1) The cells on the patterns were fixed with 4% formaldehyde in PBS (formal saline) for 20 minutes at room temperature.

2) They were then rinsed very gently with PBS.

3) Without allowing the coverslips to dry out, they were transferred to the solution containing the anti-laminin antibody (a 1:35 dilution of the stock solution from Sigma in PBS containing 0.5% BSA), and incubated for 30 minutes at room temperature.

4) The coverslips were then very gently rinsed twice in PBS containing 0.5% BSA.

5) The coverslips were incubated with the anti-rabbit antibody coupled to peroxidase (in a solution prepared in the same way as the anti-laminin antibody) for 30 minutes at room temperature, and then washed with two changes of PBS.

6) Finally, the DAB assay was carried out as described in the section dealing with peroxidase patterns.

RESULTS

NEURITE OUTGROWTH ON UNPATTERNED SUBSTRATES

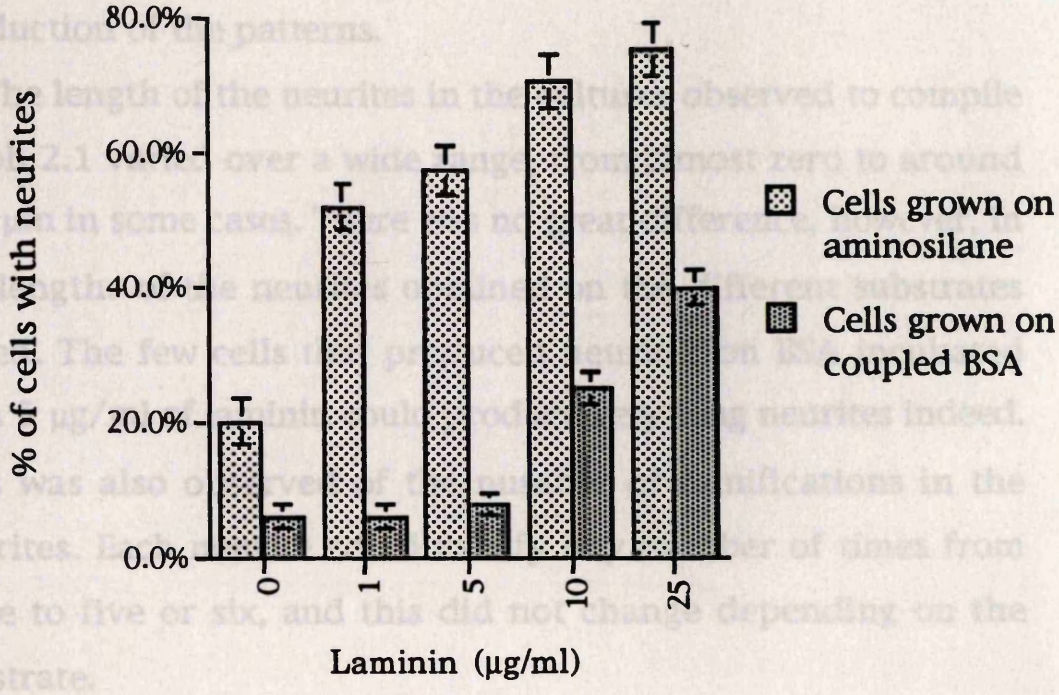
As a first step towards assaying the ability of laminin patterns to guide neurite outgrowth, experiments were carried out in which nerve cells were cultured on variously treated glass coverslips, but without any pattern on them. The different treatments were devised to make each coverslip's surface composition similar to one of the different areas that would eventually comprise the surface of the patterns. Culturing cells on these coverslips would thus show whether there was any difference between those surfaces in their neurite outgrowth promoting properties, how big this difference was, and indeed, whether they promote neurite outgrowth at all. Furthermore, these experiments helped to adjust the concentrations of the reagents used to treat the slides, so that the difference in promoting neurite outgrowth between the different surfaces would be maximised.

All the steps mentioned here are identical to those described in the Methods sections dealing with protein patterning.

One set of coverslips was treated with aminosilane, glutaraldehyde, BSA, ultrasonicated in acetone and then incubated with different concentrations of laminin. This treatment should mimic that received by areas in the pattern that would end up covered by BSA. Another set of coverslips was treated just with aminosilane and then incubated with different concentrations of laminin. These should mimic the areas in the patterns which would be covered by laminin.

Nerve cells from chicken DRGs were cultured on these coverslips for 24 hours, then fixed and observed in a phase contrast microscope. The percentage of cells producing neurites

on each surface was counted and plotted in graph 2.1 (only nerve cells displaying neurites longer than twice their own cell body diameter were counted). Each value in the graph represents the average of four cultures in duplicate, counting at least 50 cells per culture.



Graph 2.1: This graph compares the percentage of cells that produce neurites on two different kinds of substrates. One of them was produced by incubating aminosilanated glass with laminin; the other was produced by incubating glass with coupled BSA with laminin. The error bars are S.E.M.s.

It can be seen from the graph that laminin adsorbed over aminosilane is by far a better substrate for neurite outgrowth than laminin adsorbed over BSA, whatever the concentration of laminin adsorbed. As has been discussed in the introduction to

the first part, the reason is probably that laminin does not really adsorb to coupled BSA, at least not in the lower range of concentrations (up to 5μg/ml). This is supported by the fact that nerve cells cultured on coupled BSA alone showed the same percentage of neurite outgrowth as nerve cells cultured on laminin (up to 5μg/ml) incubated over coupled BSA. Thus, it was decided to use laminin at a concentration of 5μg/ml for the production of the patterns.

The length of the neurites in the cultures observed to compile graph 2.1 varied over a wide range, from almost zero to around 400 μm in some cases. There was no great difference, however, in the lengths of the neurites obtained on the different substrates tested. The few cells that produced neurites on BSA incubated with 5 μg/ml of laminin could produce very long neurites indeed. This was also observed of the number of ramifications in the neurites. Each neurite could ramify any number of times from none to five or six, and this did not change depending on the substrate.

CELLS CULTURED OVER PATTERNED LAMININ

Seeing that the surfaces that would comprise the patterns had a markedly different effect upon the production of neurite outgrowth by DRG cells, some patterns were made and tested for their ability to guide neurite outgrowth. The first pattern tested consisted of a background of BSA with small circuits of laminin (see fig 2.4). These were patterns that had been devised to produce microcomponents for electronic circuits, and were used here only as a first test, to see whether growth cone behaviour would be affected by a laminin pattern. DRG cells were cultured

Fig. 2.4: Photo-montage of phase contrast micrographs of DRG cells growing over a laminin circuit. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark. Bar: 100 μm.

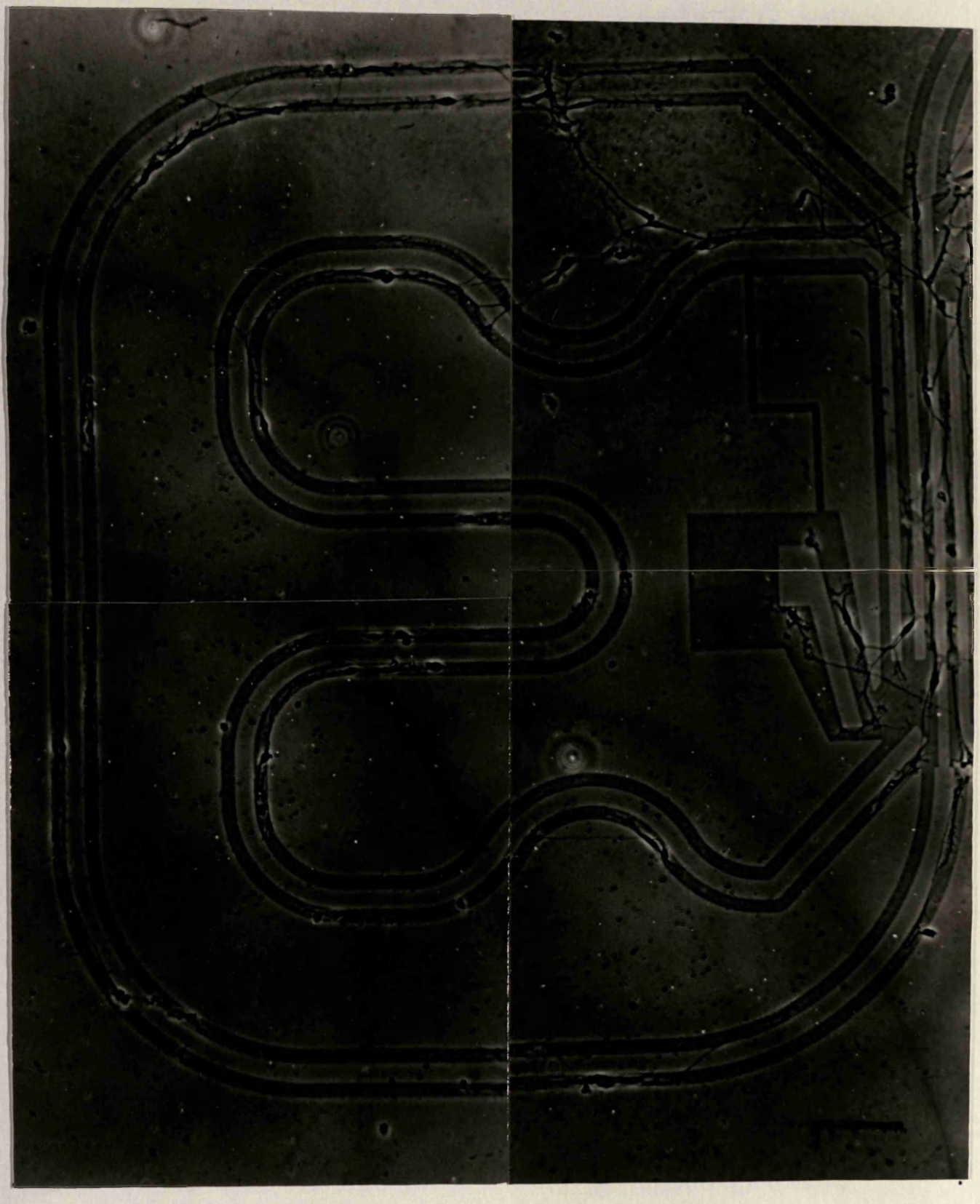


Fig. 2.4

Fig. 2.5: Nomarski optics micrograph of a DRG cell growing over a pattern of laminin. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark. Note how the neurite crosses over the stripes devoid of laminin. Note also how the filopodia in the growth cone are longer than the width of the laminin free areas. Bar: 10 μm.

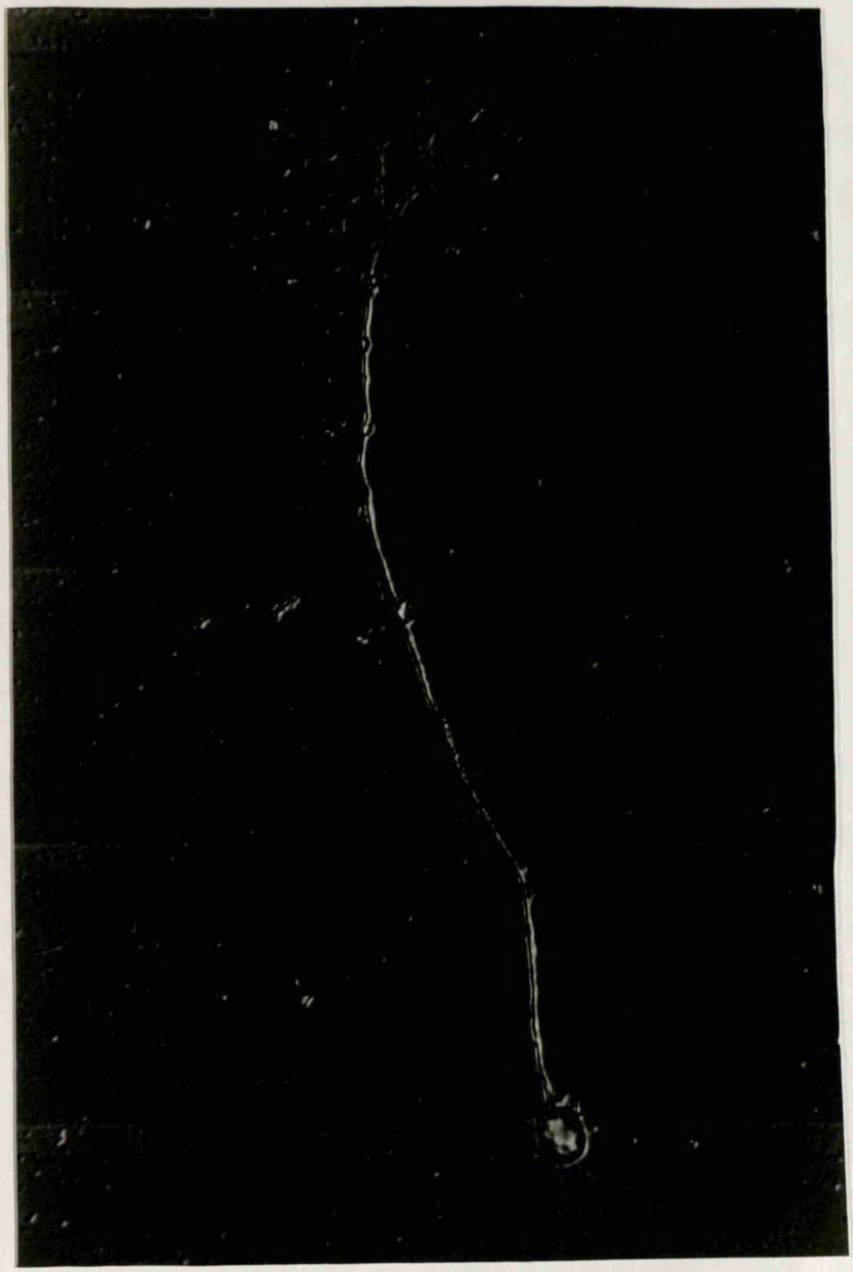


Fig. 2.5

Fig. 2.6: Phase contrast micrograph oh a DRG cell growing over a pattern of laminin. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark Note that the neurites appear to be oriented by the laminin free areas. Bar: 10 μm.

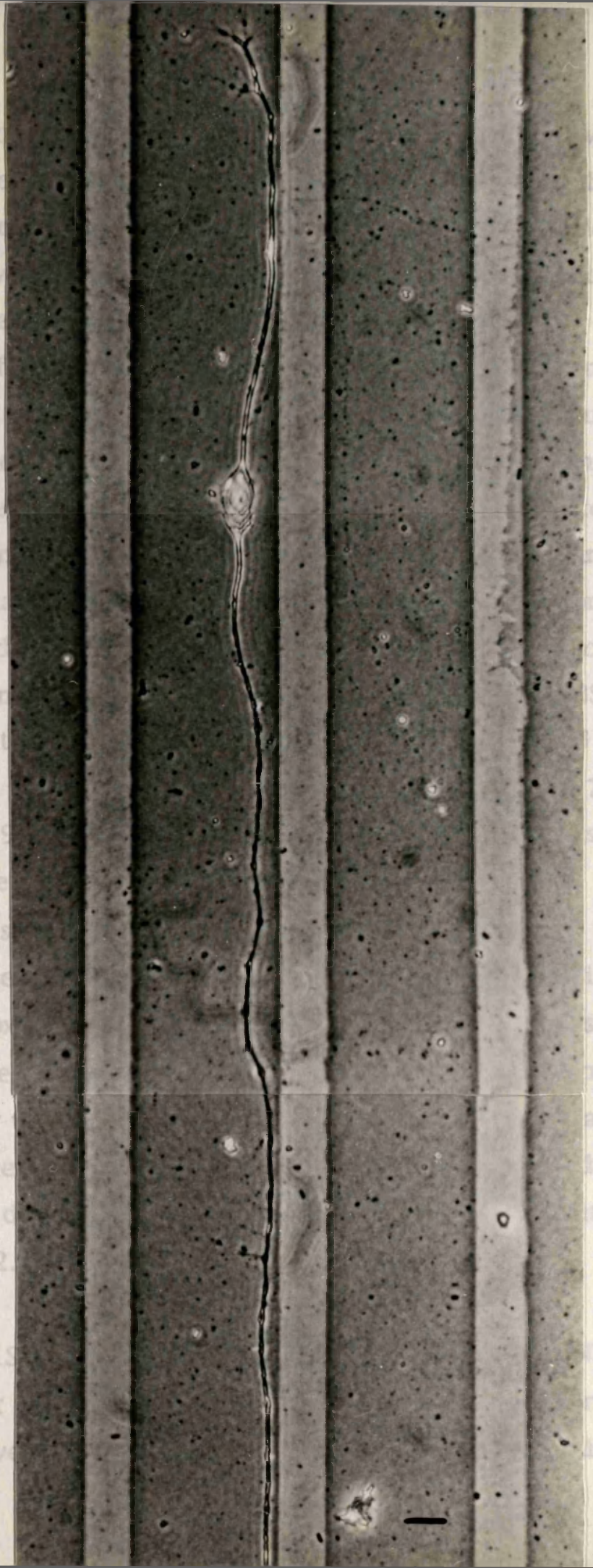


Fig. 2.6

on coverslips with this pattern for 24 hours, fixed with formal saline and stained with immunoperoxidase (this was the standard experiment performed with all different patterns described in this work). As can be appreciated on fig. 2.4, the behaviour was indeed affected.

After that first positive result, a number of original patterns were devised, with the aim of investigating the parameters that influence the accurate guidance of neurite outgrowth by laminin patterns. The first parameter that was investigated was the minimum necessary distance between the separate laminin areas, so that neurites growing on an area would not cross onto another. For this, a pattern was made that consisted of a background of laminin crossed by sets of parallel straight lines of BSA. Each set had twenty lines, with thicknesses ranging from 1.3 to 10.2 μm (1.3, 1.6, 2.0, 2.5, 3.0, 3.5, 4.1, 4.5, 5.0, 5.5, 6.0, 6.6, 7.0, 8.0, 8.4, 8.8, 9.8, 9.8, and 10.2 μm), and the laminin stripes separating these lines were an average of 30 μm wide.

As can be seen in fig. 2.5, the laminin pattern was indeed present in these coverslips, and was made visible by the peroxidase. However, the majority of the neurites present in these cultures had ignored the BSA lines and had crossed them over whenever they had encountered them. Only a very small percentage, less than 1%, were clearly guided by the BSA lines and did not cross over them. An example of this can be seen in fig. 2.6.

CELLS CULTURED OVER STRAIGHT TRAPEZOIDAL LINES

It was suspected that the inability of the pattern described above to guide neurite outgrowth was due to the thinness of the

BSA lines separating the laminin lines, and that a thick enough BSA line could prevent growth cones crossing it over. To test this, and to work out exactly how thick a line of BSA had to be to be able to guide growth cone locomotion, a new pattern was tried. This pattern consisted of a number of long trapezoidal areas of BSA separated by equally long and trapezoidal areas of laminin. A schematic diagram of this pattern is depicted in picture 2.7.

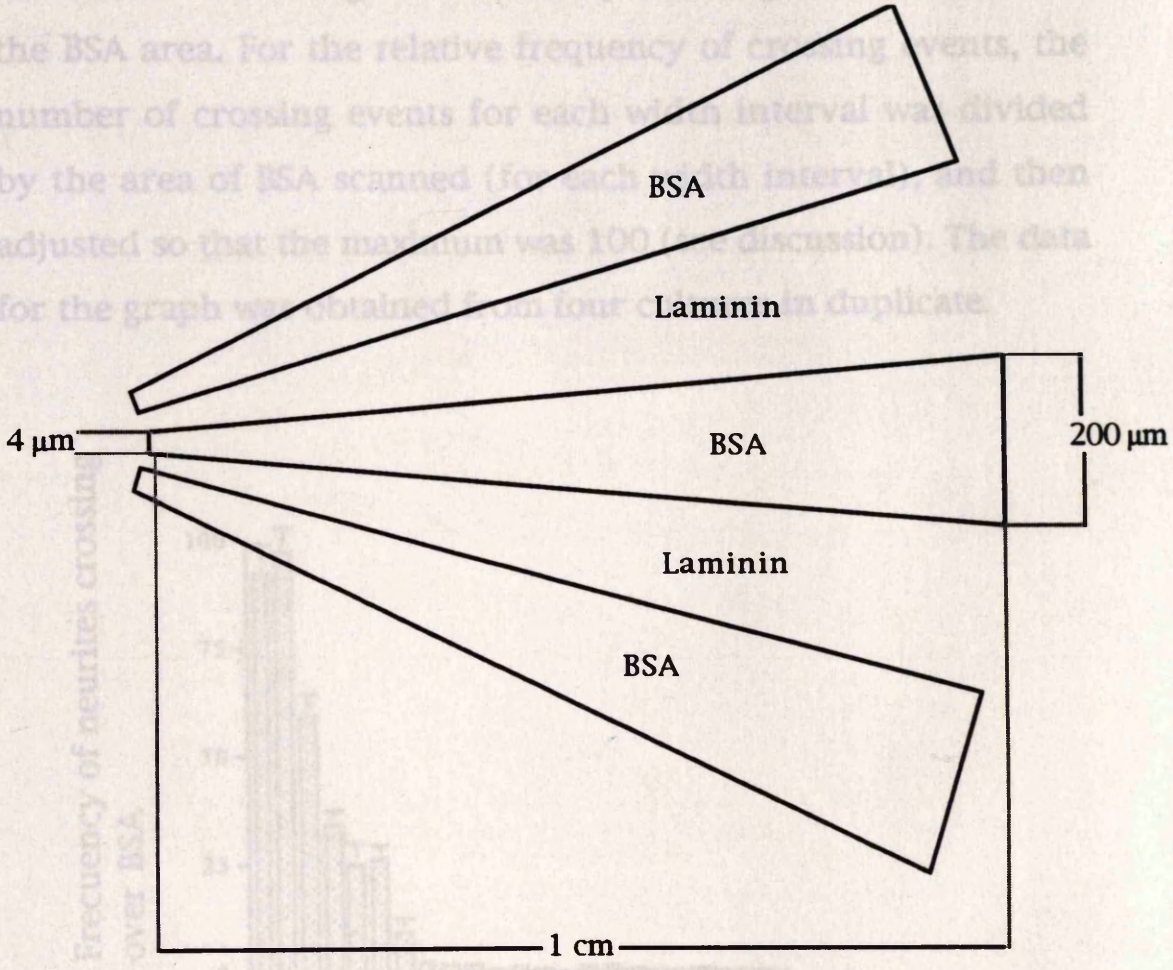
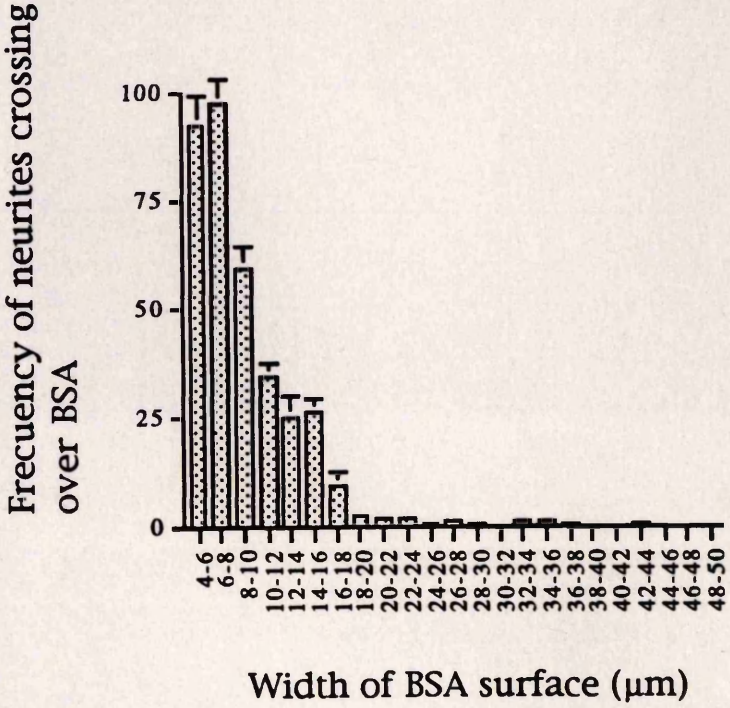


Fig. 2.7: A schematic representation of the pattern consisting of trapezoidal lines. When drawn to scale, 32 BSA lines actually fit where here only 3 are shown.

Cells were cultured over coverslips with this pattern for 24 hours, and then fixed and stained with the immunoperoxidase method. The result was that neurites were seen to cross over the BSA where the stripes were thinnest, and to be guided by the wider areas of BSA. Examples of this can be seen in figs. 2.8-2.10. This result is represented in graph 2.2. In that graph, the relative number of times that any neurite was seen to have crossed over an area of BSA of a given width is plotted against the width of the BSA area. For the relative frequency of crossing events, the number of crossing events for each width interval was divided by the area of BSA scanned (for each width interval), and then adjusted so that the maximum was 100 (see discussion). The data for the graph was obtained from four cultures in duplicate.

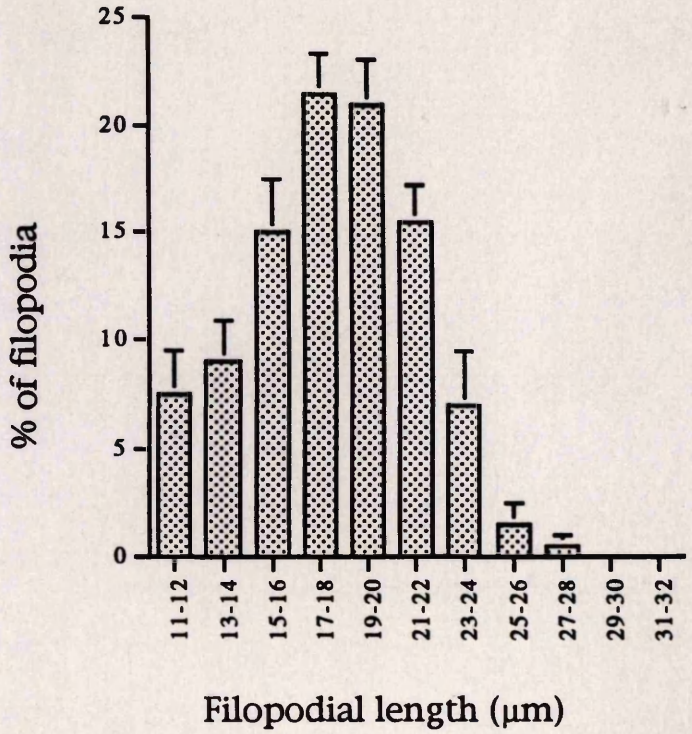


Graph 2.2: Relative frequency with which neurites cross over BSA areas of different width. The error bars are S.E.M.s.

Fig. 2.8: Nomarski optics of a growth cone from a DRG cell over a pattern of laminin. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark. Note that that several of the filopodia span the laminin free stripes. Bar: 40μm.

Fig. 2.8

As the graph 2.2 shows, BSA extensions 20 μm wide and over were quite effective in preventing the neurites from crossing over them. To see whether this number could be related to the filopodia in the growth cones, the length of these was measured. The measurements were made in the growth cones of cells that



Graph 2.3: Distribution of the lengths of filopodia from growth cones moving over unpatterned laminin. The error bars are S.E.M.s, and the means were obtained by averaging the values for each interval from each of the 6 cultures observed.

had been in culture for 24 hours, over aminosilanated coverslips coated with laminin. Only the longest filopodium of each growth cone was measured, to avoid counting filopodia that were growing or shrinking at the moment of fixation. The result of these measurements can be seen in graph number 2.3. In that graph, the percentage of filopodia with lengths falling into a

Fig. 2.9: Phase contrast micrograph of DRG cells growing over a laminin pattern consisting of trapezoidal stripes. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark. Observe that the neurites are oriented by the thickest sections of the stripes but ignore the thinner ones. Bar: 100 μm.

Fig. 2.9

Fig. 2.10: Phase contrast micrograph of DRG cells growing over a laminin pattern consisting of trapezoidal stripes. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark. In this case the stripes are all wide enough to guide the outgrowing neurites. This picture was taken from the same culture as fig. 2.9. Bar: 100 μm.

Fig. 2.10

given length interval, is plotted against length intervals. 6 cultures were observed, counting the filopodia of 20 growth cones in each culture.

The mean length of the longest filopodia in each growth cone was19.5 ± 3.4 μm. This suggests the possibility of filopodial length being the factor that limits the maximum width of a non-adhesive substrate section that a growth cone can cross over.

CELLS ON STRAIGHT THIN LINES

The next pattern that was designed to test the conditions that a laminin pathway has to fulfil to guide neurite outgrowth effectively, consisted of thin straight lines of laminin separated by broad areas of BSA. The width of the laminin lines ranged from 1 to 10 μm, and the stripes of BSA separating them were 40 μm wide, which, in view of the results of the previous experiment, should be enough to prevent the neurites from crossing them. This pattern was designed to evaluate whether it was possible to produce, with the method employed here, a line of laminin that was too thin to guide neurite outgrowth, and to see how thin such a line had to be.

After producing coverslips with this pattern and culturing cells on them, it was found that even the thinnest (approximately 1 μm) lines that could be made with the method used in this work are able to guide the growth cones of chicken DRG. Some examples of this can be seen in figs. 2.11 A-E.

It was also noted in these cultures that the growth cones on the thinnest lines had a different morphology than growth cones on unpatterned laminin. In particular, the number of filopodia per growth cone seemed to be smaller. To test whether this was

Fig. 2.11: Nomarski optics micrographs of DRG cells growing over laminin patterns consisting of thin laminin lanes over a backgroung of laminin-free substrate. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark. The widths of the lanes over wnich the neurites grew are aproximately 6 μm for **A** and **B**, 1.5 μm for **C** and **D**, and 1 μm for **E**. When growing along the wider lanes, as in **A**, the growth cones are restricted to the areas covered with laminin, with only the filopodia venturing into the laminin-free areas. Sometimes, however, the growth cones are wider than the laminin areas even on 6 μm wide lines, as in **B**. Over the thinnest laminin lines the growth cones are almost always wider than the lines, as in **C-E**. Note that growth cones over the thinnest lines tend to have a smaller number of filopodia. Bars: 50 μm.

Fig. 2.11 A

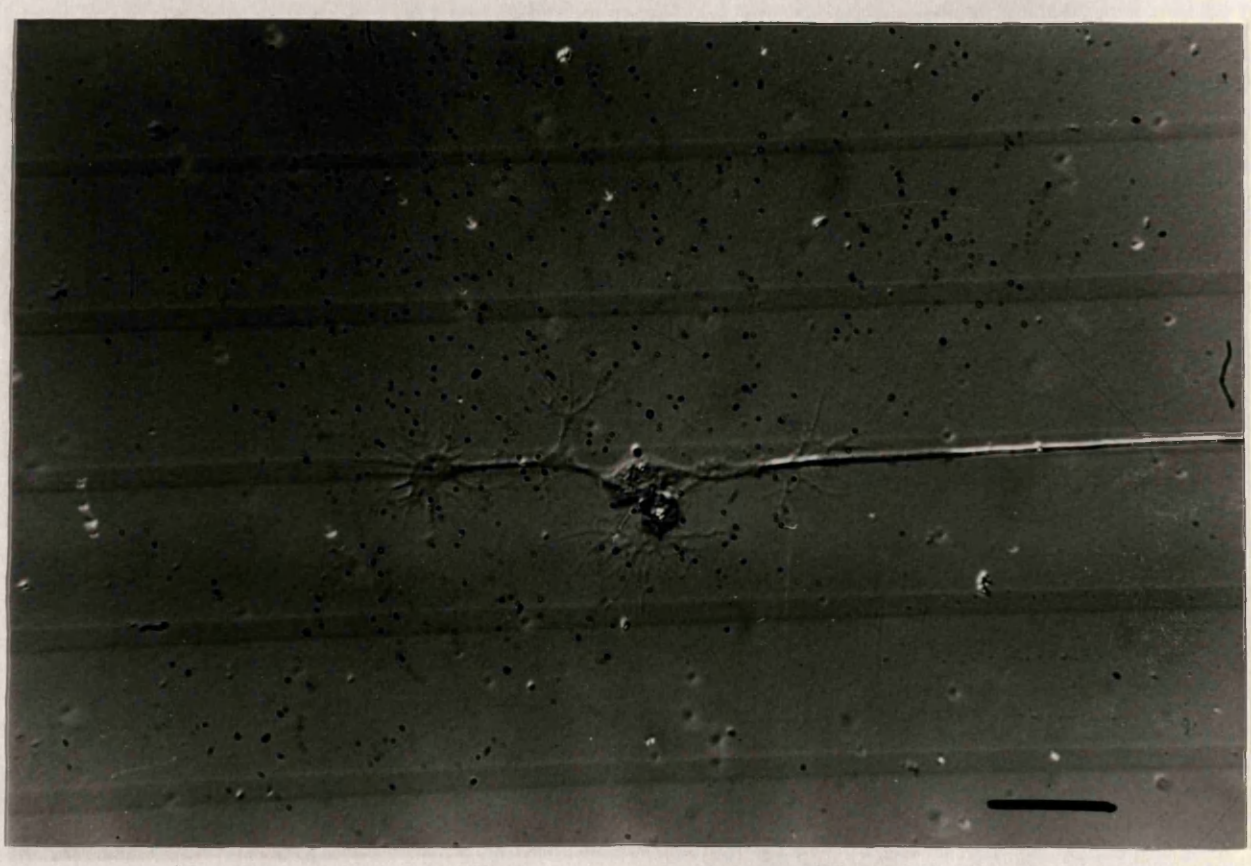


Fig. 2.11 B

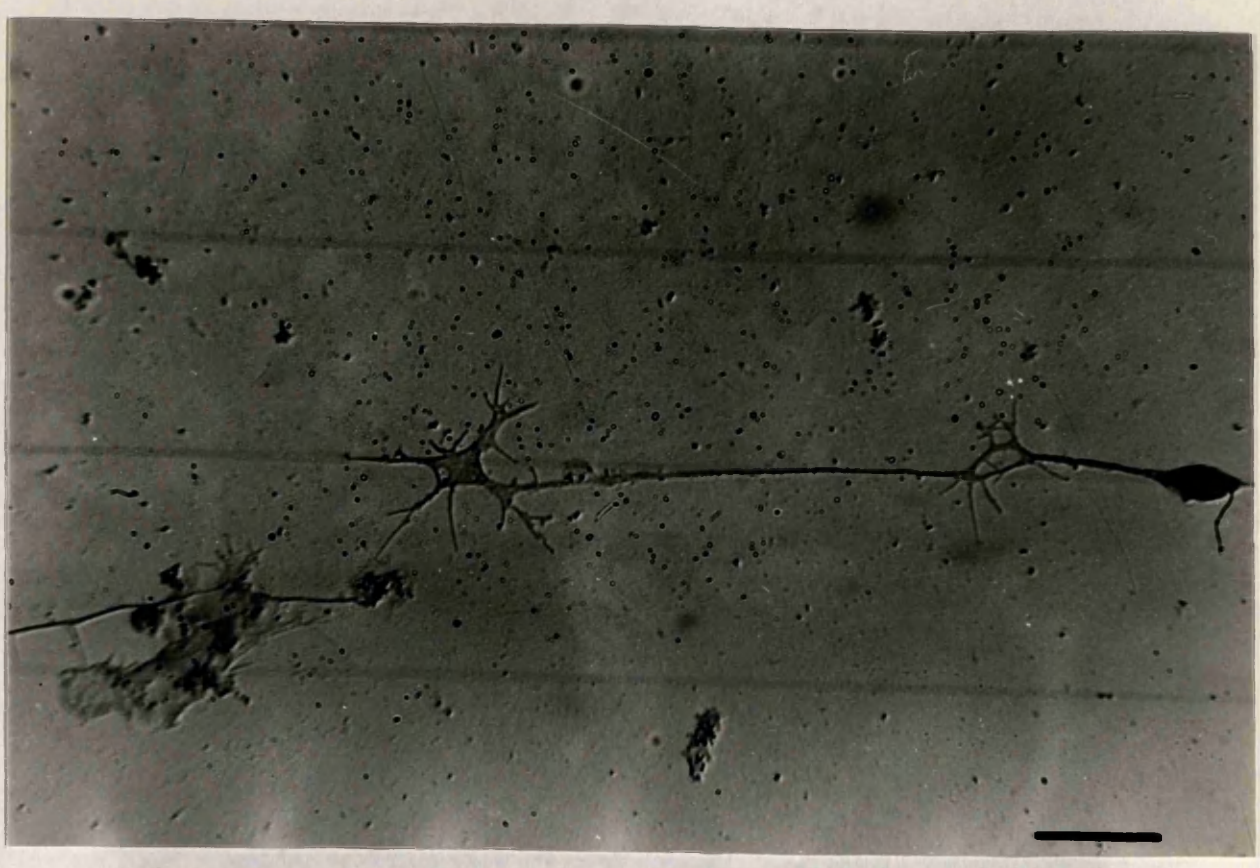
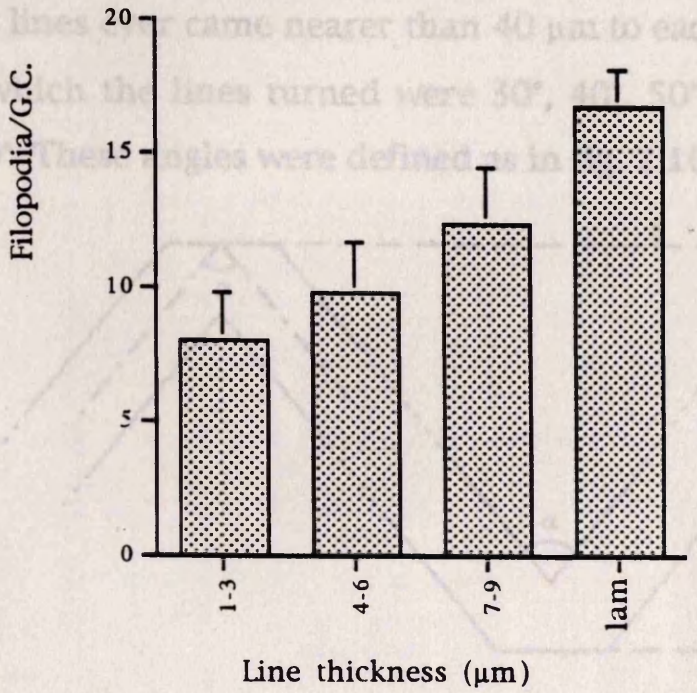


Fig. 2.11 C

Fig. 2.11 D

Fig. 2.11 E

in fact true, the number of filopodia in each case was quantitated. The result of those measurements can be seen in graph 2.4.



Graph 2.4: This graph shows the difference in the number of filopodia of growth cones over laminin lines of different thicknesses. Lam stands for unpatterned laminin. The error bars are S.E.M.s. Only alternate columns are significantly different from each other.

The data for this graph was obtained from 4 cultures for each line thickness, counting the filopodia of 20 growth cones in each culture.

As can be appreciated from the graph, the number of filopodia was indeed least on the thinnest laminin lines. The mean number of filopodia per growth cone for each line thickness was: 16.8 ± 1.4 for plain laminin; 8 ± 1.8 for 1-3 μm thick lines; 9.8 ± 1.9 for 4-6 μm thick lines; and 12.4 ± 2.1 for 7-9 μm thick lines.

CELLS ON ZIG-ZAG LINES.

Finally, a pattern was devised that consisted of zig-zagging lines of laminin on a background of BSA, in which the lines zig-zagged with different angles. Each line was 20 μm wide, and no two lines ever came nearer than 40 μm to each other. The angles at which the lines turned were 30°, 40°, 50°, 60°, 70°, 90°, and 110°. These angles were defined as in fig. 2.16.

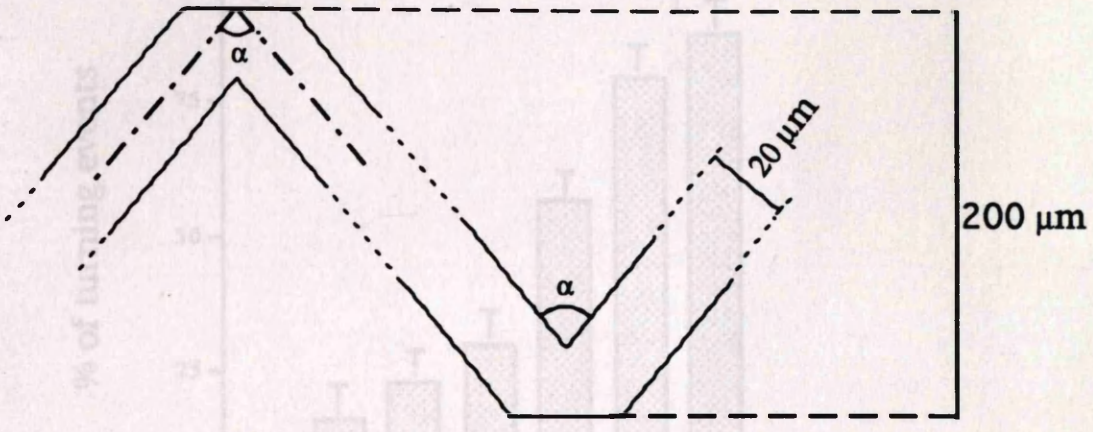
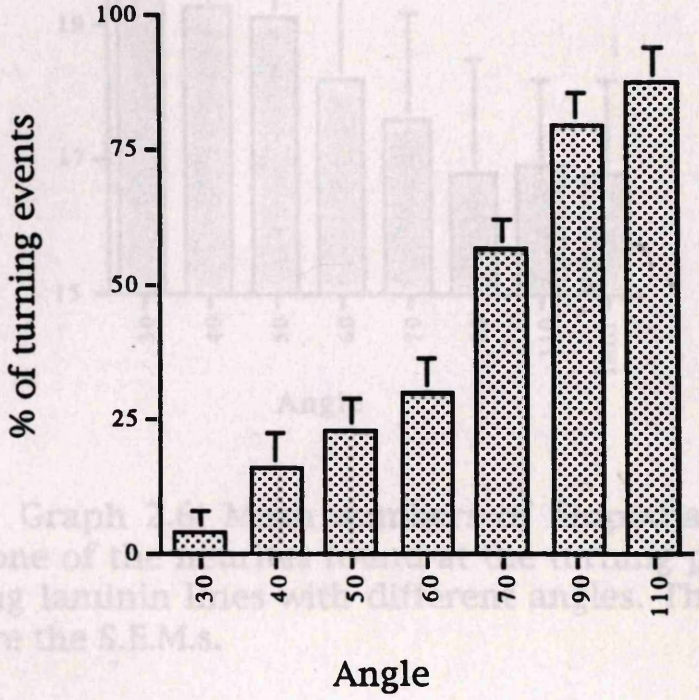


Fig 2.16: Schematic diagram of a zig-zag line, where α is the angle at which it turns.

This pattern was designed to study whether there was any limitation on the angle of the turns of a laminin pathway that can guide an outgrowing neurite. DRG cells were thus cultured over coverslips with this pattern, and fixed and stained after 24 hours in culture. These cultures showed that at least some neurites were able to turn on all the angles that were made (see figs. 2.17-2.21). However, it was noted that they turned much more often over the broader angles, whereas, on the lines with the sharpest angles, growth cones were usually found at the end of a straight section ignoring the bend, and very seldom were seen to have turned. Seeing this, the number of growth cones found at

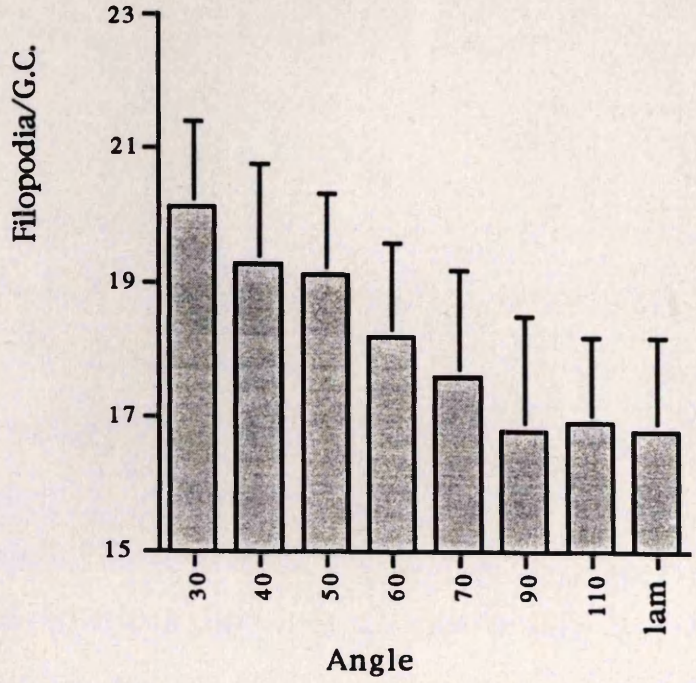
the end of a straight section, and the number of turning events, were counted for each angle. 20 cases on each of 5 cultures were counted for each angle. The result, on graph 2.5, is expressed in terms of the percentage of neurites that turned instead of remaining at the corners.



Graph 2.5: Percentages of neurites that turned when encountering each angle. The error bars are the S.E.M.s.

Another observation made in these cultures was that, as in the case of thin laminin lines, the morphology of the growth cones that were not turning at a corner was slightly unusual. In this case, they seemed larger and with more filopodia than growth cones elsewhere (for example, see figs. 2.22-2.25). To test this observation, the filopodia in the growth cones that were stalling at the bends in 4 different cultures for each angle were counted.

the result is depicted in graph 2.6, as the mean number of filopodia per growth cone for each angle tested.



Graph 2.6: Mean numbers of filopodia per growth cone of the neurites found at the turning points of zig-zag laminin lines with different angles. The error bars are the S.E.M.s.

Fig. 2.13-21: Nomarski optics micrographs of DRG cells growing over a laminin pattern consisting of zig-zagging laminin lines over a backgroung of laminin-free substrate. The laminin was stained with immunoperoxidase and the diaminobenzidine assay, and appears dark. 2-13 The angles of the turns are, in this case, 90°, and as can be apreciated in the picture, neurites were quite proficient in turning round these bends. Note how, at the turns, the tension pulls the neurite out of the laminin substrate. 2-14: A neurite that has turned round a 30° angle. Neurites turning through this angle were quite rare. Observe how the neurite has a short truncated sidebranch at the point of turning. The bent section of the neurite is not taking advantage of the full laminin area available; this neurite could obviously have turned round a sharper angle.

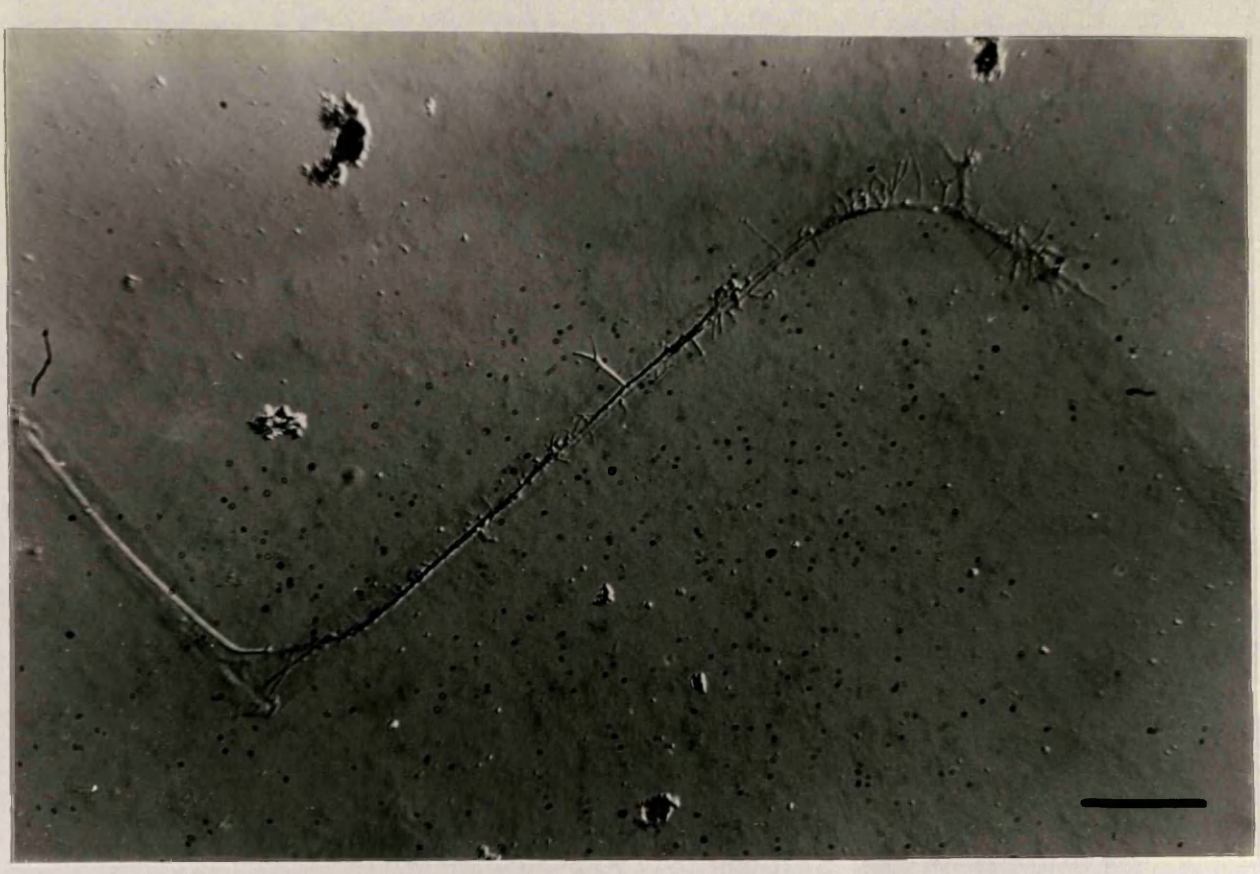


Fig. 2.13

Fig. 2.14

Fig. 2-15, 2-16: Neurites turning on lanes with angles, respectively, of 40° and 60°. In both cases, the neurites have produced a sidebranch at the bend in the lane. Only the branch following the laminin lane elongates, whereas the other stays put at the bend.

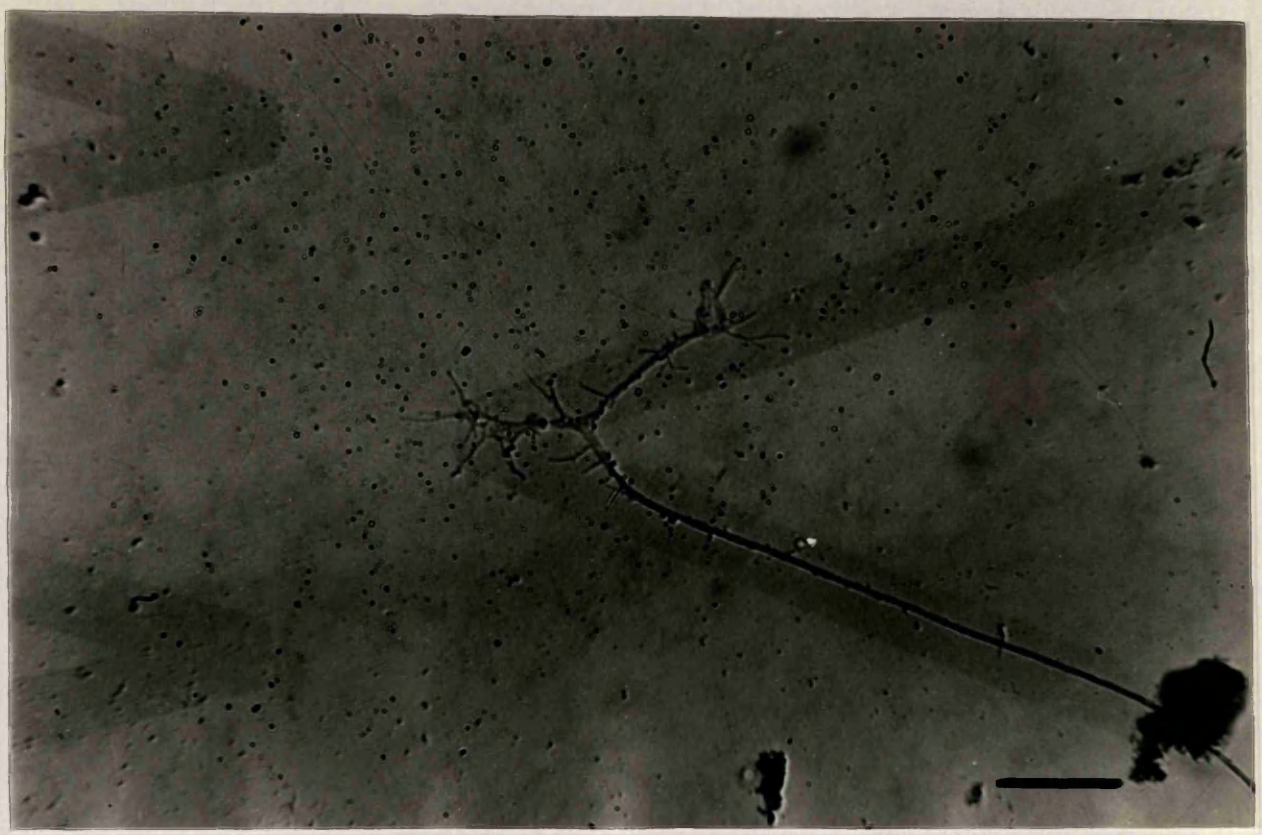


Fig. 2.15

Fig. 2.16

Fig. 2-17: A neurite has turned round a bend with an angle of 70°. In this case, it has not produced a sidebranch at the bend, and without that to act as an anchor, the tension has pulled it out of the laminin area. The displacement was in some cases larger, and in some cases smaller, as in A. Note the thin spines linking the displaced section with the path followed by the growth cone. **2-18-21**: Pictures of some cells, the growth cones of which have reached a bend in the laminin pathway. The angles in the bends are, respectively, 30°, 30°, 50° and 60°. In general, the area of these growth cones is enlarged, and the number of filopodia larger than normal. Bars: 50 μm.

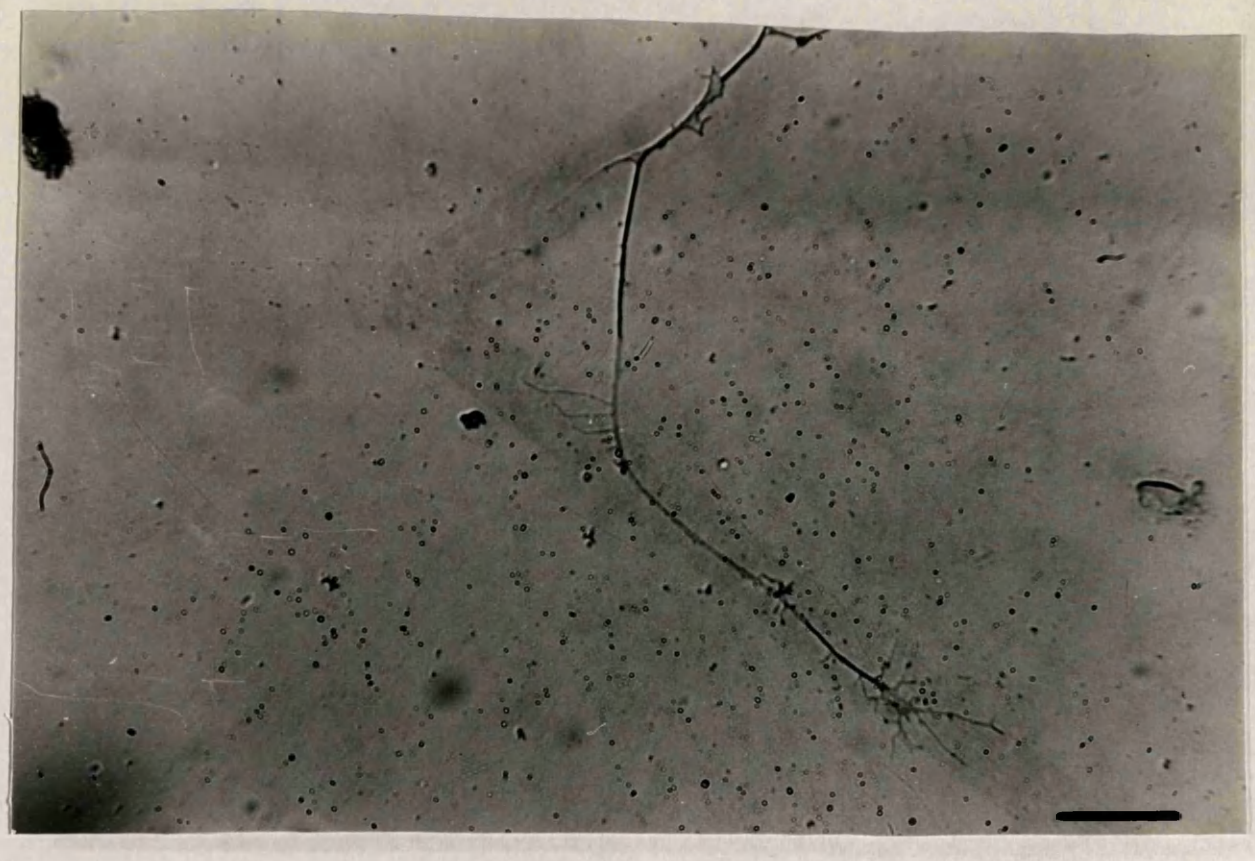


Fig. 2.17

Fig. 2.18

Fig. 2.19

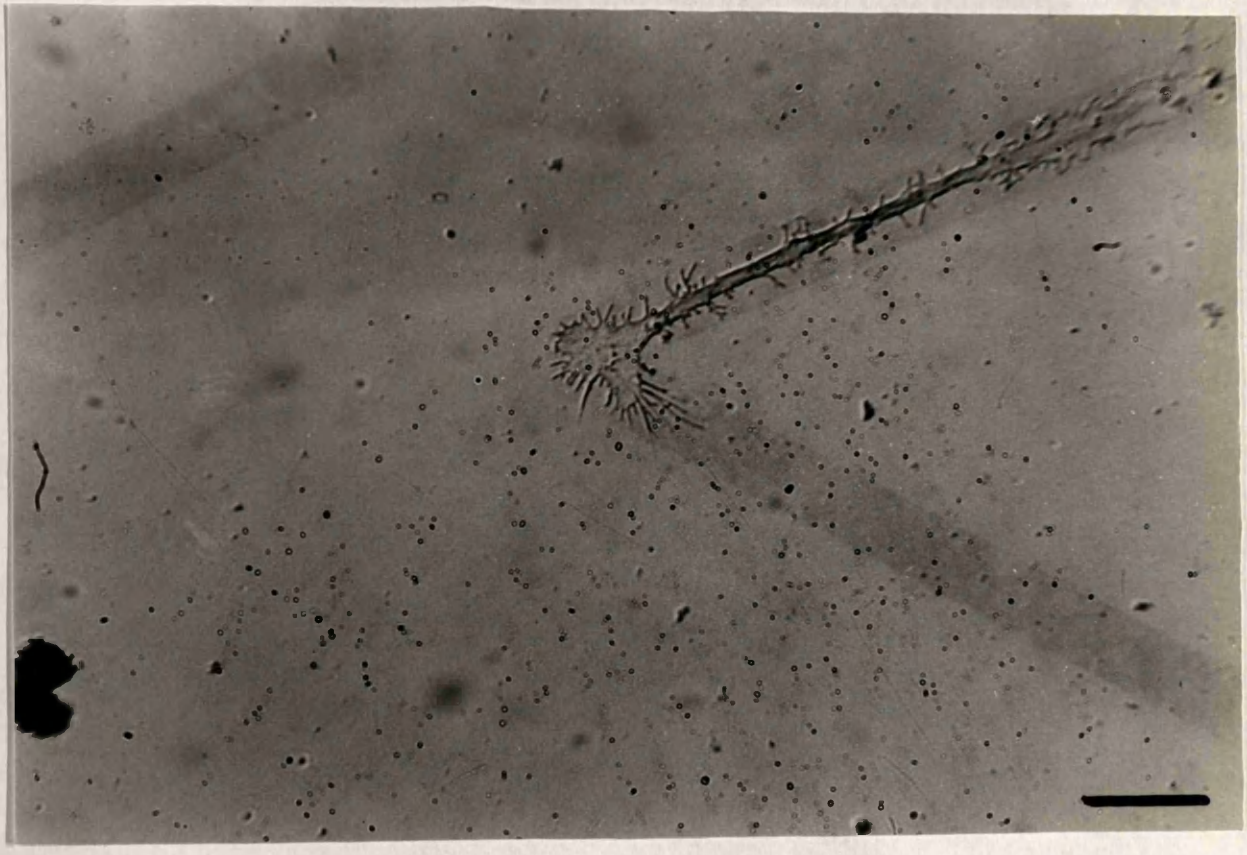


Fig. 2.20

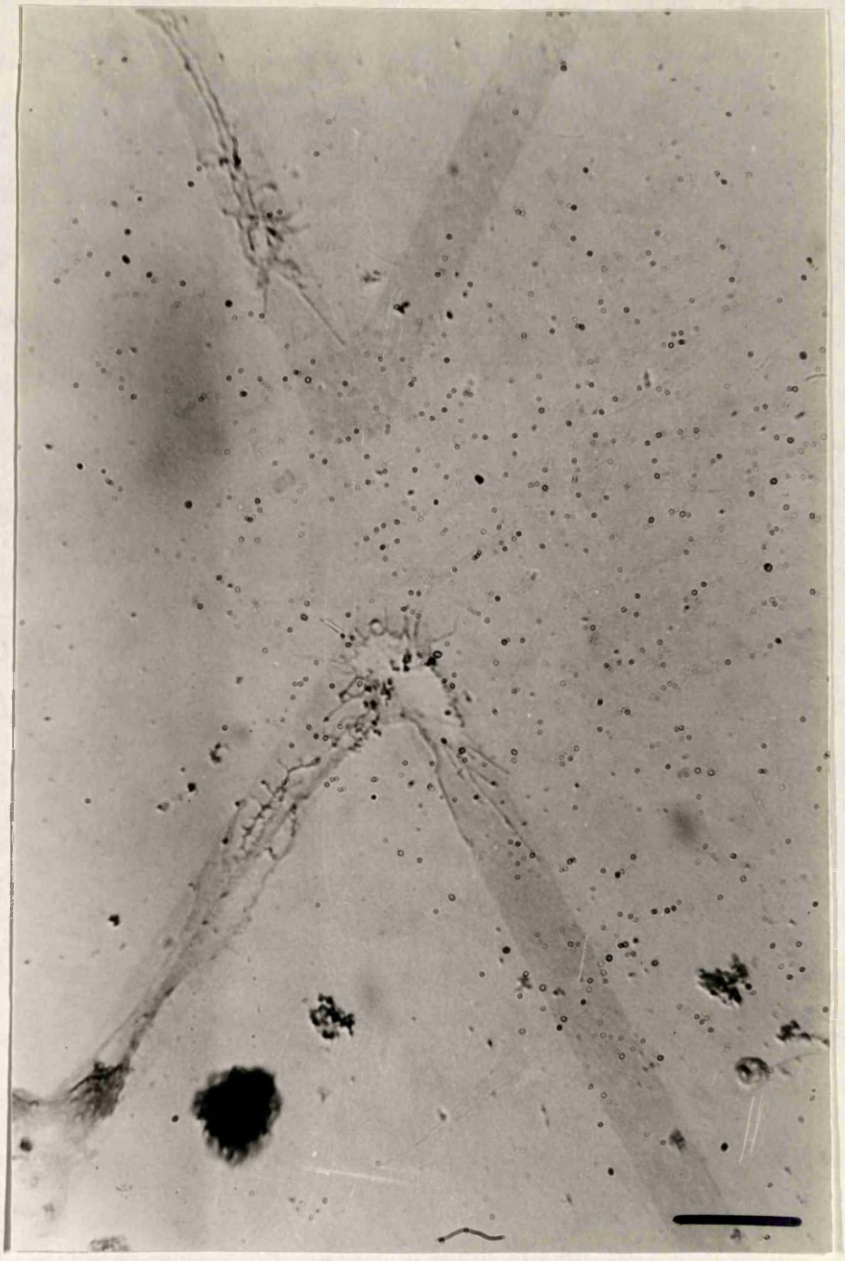


Fig. 2.21

DISCUSSION

In order to achieve the goal of this part of the project, i.e. the guidance of neurite outgrowth *in vitro* by means of micropatterned laminin, I started by studying the response of nerve cells cultured over unpatterned substrates. These substrates were the result of incubating plain aminosilanated glass, and aminosilanated glass coupled to BSA, with laminin. The reason for this choice of substrates was that they should mimic the surface composition of the different parts that would eventually comprise the patterns. Thus, the behaviour of nerve cells cultured on them should show how cultured cells would respond on the different areas of the patterned substrates.

It is plain, from graph 2.1, that these different substrates elicit different responses from cultured DRG cells. The main difference, which is the one shown in the graph, is the percentage of cells stimulated to produce neurites. On the other hand, the length and the number of ramifications of the neurites produced on each substrate did not seem to be different.

From the beginning, it was assumed that the different response of the cells to the different substrates was due to the inability of laminin, at low concentrations (up to 5 μg/ml), to adsorb to glass with coupled BSA. This was later confirmed by immunoperoxidase staining of laminin on the different substrates, that showed laminin present on aminosilanated coverslips but not on those covered with BSA. At higher concentrations, laminin did adsorb to BSA: a higher percentage of cells would produce neurite outgrowth, and immunoperoxidase staining showed the presence of laminin on the surfaces. This could in part be due to the ability of laminin molecules to interact with each other and produce ordered networks (Yurchenco and

Cheng, 1993). If a few laminin molecules adsorbed to the BSA, they could serve as anchors for an extended network of laminin. Laminin adsorbed strongly to aminosilanated glass, and a high percentage of cells cultured over the resulting surface produced neurite outgrowth. This was somewhat expected. Rogers et al. (Rogers, et al., 1983) recommended adsorbing polylysine to glass prior to adding laminin, to culture nerve cells; other authors recommend polyornithine instead (Letourneau, 1975). The main characteristic of all the recommended substrates for adsorbing laminin is their high density of amino groups. Thus, it seemed reasonable to assume that aminosilanated glass would make a good surface for laminin adsorption, and for exposing the neurite outgrowth promoting moieties of laminin.

Another fact that can be deduced from the low percentage of cells producing neurites on coverslips with BSA is that BSA also prevents the adsorption of serum proteins. Serum contains fibronectin, thrombospondin, and other proteins that promote neurite outgrowth (Neugebauer, et al., 1991, Rogers, et al., 1986), and is included in the growth medium used in this work. To avoid interference, the percentage of serum in the medium was kept low (2%).

The fact that some cells kept producing neurites on BSA even when no laminin was present could nevertheless indicate that traces of serum proteins were being adsorbed onto the BSA. Alternatively, it could indicate the existence of a subpopulation of DRG cells that need little or no adhesive proteins on the substrate to produce neurites. This latter explanation could be supported by the observation that the length of the neurites is very similar both on laminin and on BSA.

Another point that could be discussed here is the choice of culturing the cells for just 24 hours. The reason for this was, on one hand, that after that time, the number of neurites and their length was enough to permit their study. On the other hand, cultures kept for longer showed a marked increase in the number of fibroblasts and glial cells. Neurites grow very well over the surface of these cells, and this interfered with the outgrowth caused by the substrate. Also, the observation of fixed cultures that had been kept growing for more than 48 hours on BSA, seemed to indicate that these cells could take on the role of the growth cones, and lead the outgrowing neurites. These cultures showed a considerable number of neurites, and almost all of them ended on the top of a non-neural cell. Yet, the neurites did not seem to have stopped growing, as could be seen by observing older cultures. Thus, one explanation could be that the growth cones of these neurites were attached to non-neural cells that were moving, and the neurites were extending to match that movement. Alternatively, it could be that the only neurites that were growing were those few that had the growth cones on the substrate, and that whenever they reached a non-neural cell they would attach to it and stop extending.

The results obtained from culturing cells over BSA and laminin seemed to suggest that the patterns had a reasonable chance of success. However, it could in principle be possible that when laminin comes into contact with the cell body of a nerve cell, it "sends" a signal into the cytoplasm that produces neurite outgrowth, regardless of the nature of the surrounding substrate. An effect similar to this has been described for fibroblasts, where a fibronectin coated bead touching the surface of a cell, produces

spreading on a substrate that would not otherwise promote spreading (Curtis, et al., 1992). This, however, is not likely to be the case with nerve cells, as was seen when they were cultured over patterned laminin. The simple fact that neurite outgrowth is guided by patterned proteins, as shown in the results section, argues strongly against that possibility, and suggests that the signals for neurite outgrowth are received and transduced in the growth cones.

NEURITE GUIDANCE ON PATTERNED LAMININ

The first attempt to guide neurite outgrowth on a laminin pattern was fairly successful, as can be seen in fig. 2.4. However, this pattern gave very little information about anything but the possibilities of the technique. It only said that this technique worked, but it gave encouragement towards producing more interesting patterns, that would be able to provide information about neurite outgrowth behaviour.

The second pattern tested, however, was largely unsuccessful. As described in the methods section, the pattern consisted of a background of laminin crossed by parallel lines of BSA 2 to 10 μm wide. Some neurites clearly steered away from the BSA lines (see fig. 2.6), but the majority of them crossed over them freely, even though immunoperoxidase staining of laminin showed that laminin was not present in the BSA lines (fig. 2.5). This finding could be explained if it was assumed that the filopodia in the growth cones do not need an appropriate substratum to extend in any one direction. As can be deduced from the description of filopodia in the introduction of this chapter, they are a kind of sensile organ to the growth cone. They protrude out of the

growth cone and extend probably in a random direction, to explore the external surroundings. Thus, the direction towards which they extend would not need to be given by any particular signal, and probably they could extend over a BSA substratum. It follows from this that the ability of a BSA line to stop a neurite from crossing it would be subject to the condition that the width of the line be bigger than the maximum length of the filopodia produced by the growth cone. This ability of the filopodia to extend across substrata with low adhesivities for the growth cones is the basis for the guidepost cells hypothesis. This hypothesis says that an important mechanism for the guidance of pioneer neurites in vivo is by means of guidepost cells, i.e., non-adjacent cells that are highly adhesive for the neurites and are surrounded by low adhesive medium (Ho and Goodman, 1982, Schubiger and Palka, 1985). Pioneer neurites would thus reach a guidepost cell, stop there, send filopodia around until one of them contacts another guidepost cell, and then follow that filopodium.

In any case, in the cultures described before, many filopodia could be seen that were longer than 10 μm, and often filopodia were seen crossing a BSA line. These observations, together with the above considerations, led to the design of the next experiment. This consisted of culturing DRG cells over another pattern, one that would give a wide range of BSA widths. This pattern, which was described in detail in the Results section (see fig. 2.7), consisted of trapezoidal lines of BSA over a background of laminin. These lines have the obvious advantage that they provide a continuous range of BSA widths, so that it is possible to deduce very accurately the maximum width of BSA that a growth cone can cross.

The disadvantage of this design is that it is not easy to compare the numbers of neurites crossing different sections of the BSA lines. One way of working these numbers out, would be to divide the number of times any neurite crosses a line by the number of times any neurite is found brushing a boundary but steering off it, within a given thickness interval. The problem with this is that in the areas were the lines are thinnest, all the growth cones will be close to a boundary, and will be counted as steering off. Another way of doing it would be to divide the number of crossings by the total number of neurites in the area scanned for each thickness. There also were practical problems with this approach. Because the area occupied by the thinnest parts of the lines is comparatively very small, it was necessary to make relatively dense cultures, so that often it was impossible to distinguish between neurites. In the end, the number of crossings in a given thickness interval was divided by the total area scanned for that thickness interval. This relies on the assumption that the distribution of cells, and therefore of neurites, was even all over the coverslips. If the cultures were moved a lot, the cells tended to accumulate in the centre of the coverslips; but with careful handling, the distribution was fairly even. Another source of inaccuracy was that a single neurite can obviously cross a thin BSA section more times than a thicker one. This means that dividing by the area (i.e., by the number of neurites) will overestimate the ability of neurites to cross the thinnest BSA section. Nevertheless, this overestimation is not great up to the point in which neurites start to be prevented from crossing the BSA lines, because the thickness of the lines at that point is still a very small fraction of the total length of most neurites.

One problem with the method for patterning laminin was that, probably due to the large number of steps and reagents needed to prepare each coverslip, the pattern in some coverslips was clearer than in others. This effectively meant that sometimes there was some laminin adsorbed where only BSA was supposed to be. This happened also in single coverslips, where some areas would be patterned very clearly and others would have the pattern somewhat blurred. Naturally, this interfered with the measurements under way, for the neurites would be seen crossing the BSA lines that stained lightly for immunoperoxidase more often than those, of the same thickness, that did not. To solve this problem, I tried to measure the absorbance of each line after staining, with a microdensitometer, so that it would be possible to discard the areas were the BSA lines tested positive for laminin. But this could only be done for very thick lines, and furthermore the positioning of the coverslips in the apparatus was not very accurate. In the end, only the cells in areas where the pattern seemed clearest to the eye were counted.

From these cultures on patterns of trapezoidal lines it was deduced that neurites from DRG nerve cells can ignore BSA widths of up to about 20 μm (see graph 2.2). This result contrasts strongly with the figures obtained by Clark et al (1992) for epithelial cells. These authors found that adhesive and non-adhesive lines repeated with a period of 2 μm were very effective in orienting MDCK cells in culture, even though each cell would span several non-adhesive lines. They suggest that this would allow these cells to be oriented by the conformation of extracellular matrix fibres. It is clear from the results above that growth cones would not be oriented by it.

To see whether this 20 μm limit is in fact due to the maximum length of filopodia in the growth cones, these were measured, in cultures over plain non-patterned laminin. One problem with this measurement is that filopodia take some time elongating until they reach their full length, and then they also take some time shrinking until they are totally reabsorbed into the growth cone. If all filopodia in each growth cone are taken into account, many will be measured when they are shrinking or growing, and their mature length will be underestimated. Thus, to correct this source of error, only the longest filopodium in each growth cone was measured. It was found from this that the longest filopodia in the growth cones had a mean length of 19.5 ± 3.4 μm. This figure is in quite good agreement with the hypothesis that led to the measurement, although of course it does not rule out that it is just a chance coincidence. It is somewhat longer than the measured maximum stretch of BSA that a growth cone can cross, but, then, the case where a filopodium would start to grow on the exact border between laminin and BSA, and would grow exactly perpendicular to the border line, must be very rare. If this hypothesis is true, it could well be possible that filopodial length is a factor that contributes to the discrimination of different nerve cell types on different pathways.

Hammarback and Letourneau (Hammarback and Letourneau, 1986) describe some experiments similar to those described above. They patterned laminin by irradiating agarose-albumin substratum through electron microscope grids and then adsorbing laminin onto it, so that laminin only adsorbed to the irradiated areas, as described in the first part of this thesis. The resulting patterns were islands of laminin surrounded by

meshworks of agarose-albumin lines, which could have several widths. Although their patterns were more limited than the ones used in this work, as the range of widths of the laminin-free areas was limited, their results are in close agreement with those described here. They found that around 50% of the neurites (they also used chicken DRG cells) could cross lines 17 to 22 μm wide, but only 6% could cross lines 27 to 32 μm wide. They also found that the average length of the filopodia in their cultures was around 12 μm, which does not agree with the observations in this work; But this could be due to the fact that they measured all the filopodia in each growth cone, instead of only the longest. They nevertheless correlate the crossing of non-adherent substrate with the length of filopodia. Their explanation for the advance of growth cones is in terms of substratum adhesivity, and they say that filopodial contact with adhesive substrata may lower the requirement for substratum adhesivity of the growth cone proper, and may support the tension of the neurite while the growth cone advances through a non-adhesive stretch.

It is likely, however, that substratum adhesivity does not play a major role in growth cone advance. Calof and Lander (Calof and Lander, 1991), for example, found that laminin and merosin promote olfactory neuronal migration, but are anti-adhesive for those cells. Also, Lemmon *et al.* (Lemmon, et al., 1992) studied the adhesive strength for nerve cells of several substrata, and the ability of the same substrata to encourage neurite outgrowth. They found that the results could not be correlated. It is more probable that the movements of nerve cells in general are governed by receptors coupled to a number of second messenger systems, together with a tendency of the motile parts to avoid

sharp changes in direction; this latter idea will be further discussed later.

Clark *et al.* (Clark, et al., 1993) found that when a growth cone encountered a boundary between laminin and a non adhesive area, the growth cone would not get right to the boundary, but would only extend up to some 6 or 7 μm away from the boundary. As a result of this, neurites established parallel to such a boundary were always seen 6 or 7 μm away from the boundary. I did not observe this. Growth cones in cultures over the patterns described above were seen to extend right to the border of the laminin area, but not farther (that is, in the case that the extension of BSA behind the border was great enough to contain their advance). Thus, their shape was often affected, to the effect that their normally irregular shape was rendered, on the edge in contact with the boundary, perfectly straight. I did not observe either any avoidance of the edge by the established neurites.

In this same paper, Clark *et al.* found that, whereas 12 μm wide stripes of non-adherent material did not prevent mouse DRG growth cones from crossing over them, 25 μm wide stripes did. This is obviously in agreement with my results. However, they also found that cells from the neural hemispheres would not cross 12 μm wide stripes. This could be due to these cells having shorter filopodia than DRG cells. They did not quantitate this, but remarked that filopodia longer than 6 μm were rarely seen.

NEURITE GUIDANCE BY THIN STRAIGHT LAMININ LINES

Another pattern that was designed to study the behaviour of growth cones consisted of straight parallel lines of laminin on a

background of BSA. This was designed to test whether there is a lower limit, within the technical capabilities of the method used in this work, for the width of laminin lines that can guide the outgrowth of neurites. The lower limit of line width that can be achieved with this method is around 1 μm; and these lines were quite capable of guiding neurite outgrowth, as can be seen in the results section.

This width, 1-2 μm, is much smaller than the normal diameter of the growth cones, which is typically between 10 and 20 μm. The growth cones in these thin lines were smaller than the average, and indeed sometimes were limited to the area covered with laminin. Nevertheless, in contrast to the growth cones confronted with a single boundary described above, most of the times they were larger than that, and occupied some of the area covered with BSA (see figs. 2.11-2.15). This could have some bearing in the argument outlined above, to the effect that adhesiveness is not the key factor that determines the neurite outgrowth properties of a substrate. In effect, these growth cones were sitting on a largely non-adhesive surface, supporting the tension exerted by the neurite, and were nevertheless advancing forward. That the growth cones adhered to a broader area than that covered with laminin also seemed to indicate that the direction towards which they were moving was not determined simply by an extension of their mass over the most adhesive areas of the substratum. It would seem more likely that the primary effect of laminin on guiding the growth cones was exerted on the filopodia, which were the only parts of the growth cones small enough to detect the borders of the lines (i.e., to receive a clear signal from a small expansion of laminin and no

signal at all from the surrounding expansion of BSA). If these assumptions are correct, the thin laminin lines would effectively have been acting as a succession of guidepost cells. The growth cones would not have been sensing them as continuous lines, but as discrete points that the filopodia were detecting a few μms ahead of the leading edge of the growth cones.

As has been pointed out above, the growth cones of neurites on thin laminin lines seemed to be smaller than usual. They also seemed to have less filopodia than growth cones moving over broader expansions of laminin. This was also noted by Clark *et al.* (Clark, et al., 1993), when they were studying growth cone morphology on micropatterned surfaces. Thus, the number of filopodia in both cases was quantitated, and it was found out that whereas the growth cones of neurites growing over laminin at large had an average number of 16.8 filopodia, those of neurites growing on 1-3 μm wide laminin lines had an average of only 8, and Increasing the thickness of the lines gave an increase in the number of filopodia per growth cone.

There could be more than one reason for this. One reason would be that growth cones, on these thin lines, move faster than on unpatterned laminin. Bovolenta and Mason (Bovolenta and Mason, 1987) studied the morphology of growth cones from retinal ganglion cells *in vivo*, and found that they had simple forms when they follow well defined common pathways, and more elaborate filopodial forms when they diverged, turned, or came to decision regions. They also observed that growth cones moved faster when they were in the first kind of position.

A possible explanation for growth cones moving faster over surfaces with this pattern could be that the only growth signals

they receive come from straight ahead. No conflicting signals from other directions would mean that the ones received are followed more readily. Also, the fact that the laminin they detect lies ahead of them, instead of on one or the other side, may account for increased speed, as will be seen in the next section.

Another reason for the small number of filopodia of growth cones over thin laminin lines could be simply that they are largely sitting over a low adhesivity substratum. According to Letourneau (Letourneau, 1979), growth cones on low adhesivity substrata have typically a narrow, rounded up morphology, as opposed to the flat and broadened form of growth cones on high adhesivity substrata. Although this explanation is feasible, it is not convincing, for it would not take into account the fact that even if sitting largely over BSA, these growth cones are constantly in contact with laminin. In any case, both explanations need not be mutually exclusive, and it is possible that a diminished extension of the growth cone, due to low adhesivity of the substratum, would effectively speed up its advance. In support of this is an observation made on cultures over trapezoidal lines of laminin. On this pattern it was not unusual to see growth cones that had just crossed a BSA line, or to see a growth cone with filopodia spanning a thin BSA line. However, in very few cases were growth cones seen in the act of crossing a line of BSA, which seems to imply that they did so very rapidly.

In principle, measuring the lengths of a number of neurites should tell whether neurites grew faster over thin lines of laminin than over unpatterned laminin. However, this was not as straight forward as it seemed at first. The reason was that a majority of cells in the cultures over thin laminin lines would

initially be placed over the BSA areas of the pattern, simply because these occupied more space than the laminin areas. Many of these cells would produce a neurite that would travel a short distance over BSA, and then encounter a laminin line and start following it. In this way, neurites growing over unpatterned laminin had a head start that was difficult to calculate. Also, it would not be correct to measure only the neurites sprouting from a cell body that was situated over a line of laminin, because it is not unusual for cell bodies to be dragged some distance by the tension of the growing neurite. Thus, not all the cell bodies located on a line had necessarily been there when the culture was set up. Furthermore, it is likely that this dragging would be more pronounced on cells over coverslips with a pattern of thin lines, for the substratum would be less adhesive for the cell bodies. Thus, further studies will be needed to test the hypothesis that growth cones travel faster if they are placed over thin lines of laminin, perhaps by time-lapse videotaping cultures on these patterns. This, however, presents the problem that without staining, the patterns are invisible, so it is quite difficult to focus on a cell that is placed on an interesting area of the pattern.

Another effect that was observed on cells that had been cultured over this pattern concerned their overall morphology. DRG cells cultured over homogeneous laminin were often multipolar, although unipolar and bipolar cells were also often observed. However, on thin laminin lines, they were always either unipolar or bipolar. When they reach maturity *in vivo*, they have a pseudo-unipolar morphology. It would seem likely that an ability to produce more neurites than needed, which can

be retracted if they do not reach their proper destination, would increase the chances of these cells connecting with their correct targets. In addition, when growing on laminin lines, neurites never branch. This would seem obvious, but it supports the notion that there are very few of the navigational decisions of mammalian neurones that are genetically pre-determined.

NEURITE GUIDANCE BY ZIG-ZAG LAMININ LINES

The last pattern that was tried in this work consisted, as described in the results section, of zig-zagging lines of laminin on a background of BSA. This pattern was designed to test the ability of extending neurites to follow turns on a laminin pathway. As indicated before, the angles at which the lines turned ranged from 30° to 120°.

The conclusion reached after studying cultures of DRG cells over coverslips with this pattern, as depicted in graph 2.5, was that neurites can normally turn through angles 110° and 90°. Sharpening the angles, however, brought a steady decrease in the number of neurites that turned, and only 4% of the neurites encountering a 30° turn would have followed the path after 24 hours in culture. Nevertheless, with all the angles tested, there always were some neurites that had turned (see figs. 2.17-2.21). The neurites that had not turned were observed to have followed one of two courses: Either they had stopped at the bend, or they had followed advancing straight ahead, across the BSA substratum.

The course that was followed more often was that of stopping at the bend. This study was made on fixed cultures, as has been stated earlier, so it is obviously not possible to state categorically

that the growth cones of these neurites had stopped moving. Nevertheless, it is most likely that they had done so, for the number of growth cones in these cultures fixed on the bends increased dramatically the sharper the bends became.

The other course, that of straying from the laminin pathway onto the BSA rather than turning, was also more common in the paths with the sharpest angles. However, the events in which neurites followed this course of action were not quantitated, and they are not included in the percentages depicted in graph 2.5. The reason for this was an effect that has already been described, namely that not all the coverslips received a perfectly sharp BSA/laminin pattern, but many coverslips had areas with some laminin present where only BSA was supposed to be, and vice versa. Thus, growth cones tended to invade more often the BSA when traces of laminin were present there. This increased tendency to extend into the BSA was triggered, specially when the neurites were on pathways with very sharp bends, by very small amounts of laminin in the BSA. A neurite extending over a straight laminin path would very seldom stray onto a BSA extension that stained very lightly for laminin, whereas a neurite the growth cone of which had reached a bend in the path, would readily extend into a BSA area with traces of laminin. For this reason, it was not possible, with the techniques available, to discard all the instances were this effect was produced, and so I decided to disregard all the occasions in which neurites crossed into BSA.

From the previous paragraph, it is obvious that the capacity of growth cones to turn at sharp angles is somewhat overestimated in this study. This overestimation, however, is small: In the

coverslips where the pattern was clearest, the times when growth cones were fixed at the turns greatly outnumbered those in which the neurites had extended straight into the BSA.

When a growth cone turned round an angle, and continued advancing onwards, it sometimes happened that the newly produced neurite did not adhere well to the laminin pathway, and thus the tension produced by the advancing growth cone pulled it out of the pathway. These cases looked, after fixing the culture, as if the growth cone had started turning before reaching the bend in the pathway (see figs. 2.17, 2.21), except that, occasionally, thin spines could be discerned linking the neurite to the pathway. This effect was also observed by Fromhertz (Fromherz, et al., 1991), studying the response of leech neurones to pathways of a leech extracellular matrix extract. These cases were all considered to be successful turnings.

In the case of the neurites following pathways that turned with the broadest angles (90° and 110°), the percentages that are depicted in graph 2.5 as not turning, around 15%, are probably an artefact of the quantitation procedure. Around 10% of the area of the laminin pathways is taken by the bends in these cases. Thus, about 10% of the growth cones of those neurites were probably at the turns at the moment of fixing by chance, simply because at some point they would have to go through there. This artefact would not affect so much the rest of the neurites, on pathways with sharper angles. As will be seen below, these neurites probably had stopped extending as soon as they had reached the bends, so that the time that they would have spent turning becomes negligible in comparison with the time they actually spent immobilised at the bend.

The neurites that had faithfully followed the pathways of laminin through the bends, were observed to have done so in two different ways. Most commonly, the part of the neurite that was bent was indistinguishable from the rest of the neurite: a thin, smooth tubular section. In some cases, however, the neurite had a small side-branch sticking out of the shaft at the point at which it was bent, pointing outwards, in the opposite direction to that towards which the neurite had turned (see fig. 2.18). This probably was an indication that at the time of turning, the growth cone had split in two, and so the neurite had branched. One of the new growth cones would have led one branch of the neurite in the new direction of the pathway, while the other would have pointed straight out of the pathway (see figs. 2.19, 2.20). Of the two new branches, only the one following the laminin pathway would have kept on growing; the other one would have stalled at the turn, serving as a sort of anchor for the rest of the neurite. Sretavan and Reichardt (Sretavan and Reichardt, 1993), studying the dynamics of growth cones from retinal ganglion cells *in vivo*, found a similar effect. When the developing neurites of these cells enter the central optic chiasm region, they have to reorient themselves, and take a new direction 90° degrees away from the former one. These authors found that in about a third of the cases, the neurites in that situation split in two, so that one branch would take the right direction whereas the other would take the opposite. After a short period of time the branch extending in the wrong direction would retract and be reabsorbed.

From the observation of the behaviour of neurites extending on zig-zagging paths of laminin, thus, it can be concluded that

they have the ability to turn over the whole range of angles that were tested. However, whereas they turn readily over the broadest angles, they rarely do so over the sharpest angles. This would seem to indicate a tendency of the neurites to extend in the straightest possible line, so that they will disregard most of the signals detected from a direction conflicting with that in which they have been consistently extending. In this respect, Katz (Katz, 1985) studied the paths followed by neurites extending over acid-washed glass coverslips. He found that, in spite of the fact that growth cones appeared to actively alternate sides -right and left from the straight line of growth-, and that the growth cones necks exhibited all possible angles, the neurites tended to grow in a straight line. He concluded that this is due to an intrinsic resistance of the neurites to bending. In the light of the results described in this work, however, this conclusion does not seem likely, because neurites could turn through all the angles present. Also, the shafts of the neurites did not adhere very strongly to the substrate, as was shown by the fact that they could be dragged by the advancing growth cones after turning through a bend (see fig. 2.21). This would mean that if a growth cone made a turn, the neurite would not necessarily have to bend itself over the pathway, but could disregard the laminin pathway and simply follow the growth cone through the straightest possible line, so no resistance to turning would effectively be observed. Thus, it would seem that the resistance to turning over sharp angles is a characteristic of the way growth cones advance. It could be that signals are more strongly amplified in certain parts of the growth cone (the distalmost parts) than in others, or that the molecular machinery that reacts

to the signals is concentrated in certain parts of the growth cone. A scenario that would explain this resistance to turning would be that in which growth cone advance is produced by removing a barrier (gelled F-actin) that was restricting the extension of the microtubule bundle (see the introduction). Obviously, this bundle, coming from the shaft of the neurite, will tend to point straight ahead, in the direction in which the neurite has been previously advancing. Thus, removing the barrier from the front of the growth cone would be much more effective in triggering advance than removing it from the back of it.

Whatever the case, a growth cone, confronted with a sharp angled turn on the pathway it was following, would stop advancing, and would stall at the turn, possibly testing the environment for signals to move forwards. A few of them, however, would take the turn. There are three possible situations that would make them to do so. One is the situation in which, while stalling, the growth cone splits in two. This is suggested, apart from the observations in the paper by Sretavan and Reichardt, by the observation of neurites extending over unpatterned laminin. These neurites are never observed to make sudden turns; however, when they branch, the branches can take almost any new direction, regardless of the direction of the original neurite. Another situation could be that in which the growth cone changes direction by small degrees, turning through a wide angle several times in succession without advancing in the meantime. This could be true if we assume that growth cones have a stereotyped way of changing the direction of advance, with a more or less fixed angle of turning. This could also be true if the scenario outlined in the previous paragraph were to be

true; in that case, a gradual change in the orientation of the microtubule bundle could be brought about by recurrent solifications of the actin meshwork, each of them reorienting the bundle of microtubules a few degrees to the side of the previous direction. The last possibility is that in which the signal from the new direction becomes momentarily so strong that it overcomes the tendency of the growth cone to disregard it. This could happen, for example, when several filopodia point in the same direction at the same time and perceive the signal simultaneously.

The last two situations are not based on any direct observation, and it could well be that the only mechanism at work when neurites turn on a sharp angle is that described first. If that were the case, however, it would be difficult to explain why the percentage of neurites turning decreases steadily if the angles are sharpened: The sharpness of the angle would not be likely to affect the speed with which a side branch moves, and there is no reason to believe that a sharp angle would discourage branching. Thus, it seems likely that at least one of the other possibilities is also real.

As has been noted before, Bovolenta and Mason (Bovolenta and Mason, 1987) observed that growth cones adopt elaborate filopodial forms when they are turning. To test whether this was the case in our cultures, I counted the number of filopodia on the growth cones that were in a bend at the moment of fixing the cultures. The average number of filopodia in the growth cones that were on a straight section of the pathways was very much the same as that of growth cones on coverslips entirely covered with laminin, i.e. around 16. The growth cones on the bends,

however, had a higher number of filopodia, and this number would increase the sharper the angles were, until those at 30° bends had an average of about 20 filopodia (see graph 2.6). This would seem to support some of the ideas outlined above: These growth cones would actually be stalling at the bends, exploring the environment with an increased number of filopodia.

In this observation there is something, however, that is not easy to explain: There seems to be no reason for an increase in the number of filopodia after the angles are sharp enough to stop the advance of the growth cones. Once a growth cone is stopped by an apparent end to the pathway it was following, it should trigger a fixed increase in the number of filopodia, and the sharpness of the angle that the path is turning through should have no bearing on the degree of that increase.

I concluded that the observation described in the previous paragraph is due to a fact that has been described above, namely that the bends occupy a certain percentage of the pathways. As was mentioned above, this would have a bigger effect in the cases of broad angles. The broader the angles are, the faster the growth cones seem to go through them, and thus the more noticeable should be the periods of time when they are entering or exiting the bends. During those periods, the growth cones will have a normal number of filopodia, so the average number of filopodia of growth cones turning through the broadest angles will be underestimated. With sharper angles, a smaller percentage of the growth cones found on the bends will be entering or exiting them, so the number of filopodia per growth cone will be more accurate, thus larger.

A final hypothesis that could be drawn from the observation of these cultures, is that growth cones, confronted with a break in the pathway they are following, not only increase the number of filopodia, but also decrease the threshold signal needed to trigger its advance. This seems to be indicated by the fact that, whereas growth cones on straight lines very rarely stray from the laminin pathway and go into BSA covered areas, they do so more often when confronted with a sharp bend in the pathway. Also, as has been said above, DRG cells on a BSA substrate extend some neurites. Although this has not been quantitated, the impression was that more neurites extended onto BSA covered areas when they had been following a truncated laminin pathway than when the cells had been directly placed over BSA.

CONCLUSIONS

In the course of this work, a new method for micro-patterning proteins has been developed and tested, and it has been proven to be effective. Furthermore, this method has been applied to the construction of laminin patterns, and these patterns have been used as substrata in cultures of nerve cells. The observation of these cultures has shown that these patterns are effective in guiding the outgrowth of neurites from the cells. Several patterns with different architectures have been used in the cultures, and a number of deductions on the behaviour of outgrowing neurites have been made. These can be summarised as follows:

A). Pathways that are capable of guiding neurite outgrowth have to be a certain distance apart from each other, otherwise the growth cones of the neurites will be able to cross from one pathway to another. In the case of DRG cells the minimum

distance is around 20 μm. This distance coincides with the average length of the filopodia in the growth cones of these cells.

B). The lower limit of the thickness of a laminin pathway that can guide neurite outgrowth is out of the resolution of the technique employed in this work, which is around 1 μm.

C). Neurites from the cells employed in this work can follow a laminin pathway that turns with an angle down to 30°, which is the sharpest angle tested in this work. However, they rarely do so. Increasing the value of the angle has the effect that neurites follow the bends more often, and they turn almost always at angles of 90° and above.

This is obviously an unfinished work, one that perhaps raises more questions than it answers. More patterns with different geometries could be devised that could help answer some of these questions. There are also two lines of experimentation that follow naturally the work described here. One is the study of the behaviour of outgrowing neurites on patterned laminin using time-lapse video microscopy. The other is the extension of these studies to other nerve cell types.

As can be seen, culturing cells over these patterns is a very effective way of studying their locomotory capabilities. The versatility of the manufacturing technique, both in terms of architecture and in terms of the possible proteins that can be patterned, should make it useful in the study of the behaviour of a great number of cell types in a variety of situations.

In addition, the observations outlined above give the basis for the design of patterns with circuits in the image of real neural circuits. Culturing nerve cell on these patterns could allow the study of the electrical properties of neural circuits *in vitro*. An

example of this is described in a paper by Syed *et al.* (Syed, et al., 1990). These authors reconstructed *in vitro* the respiratory central pattern generator of the mollusc *Lymnaea*. This circuit, however, consists of only three cells, and they did not need any protein pattern to reconstruct it. Larger circuits, however, would certainly require some way of ordering neurite outgrowth.

APPENDIX: MATERIALS.

4-Aminoantipyrin, from Sigma.

N-(2-Aminoethyl)-3-aminopropyltrimethoxysilane,from Pierce.

Bottenstein N-2 supplement (x100), from Gibco.

Bovine serum albumin, >98%, essentially fatty acid free, from Sigma.

3-3'-Diaminobenzidine, from Sigma.

Dimethyldichlorosilane, from Pierce.

Glutaraldehyde, 25% solution, for electron microscopy, from FSA Laboratory Supplies..

Goat polyclonal anti-rabbit IgG antibody, affinity isolated, from Sigma.

Horseradish peroxidase type I, salt free, from Sigma.

Iodobeads™, from Pierce.

Laminin, from basement membrane of Engelbreth-Holm-Swarm sarcoma, from Sigma.

Microposit developer (for the photoresist), from Shipley.

Na ^{125}I, from Amersham.

Nerve Growth Factor from mouse submaxilary glands, from Sigma.

Nutrient mixture Ham F-12, Hepes modification, from Sigma.

S-1400-17 Photoresist, from Shipley.

Rabbit polyclonal anti-laminin antibody, affinity isolated, from Sigma.

Rhodamine B isothyocyanate from Sigma.

Sodium cyanoborohydrade, from Sigma.

Sodium [^{3}H] cyanoborohydrade, from Amersham.

Sodium [^{3}H] cyanoborohydrade, from Amersham.

Trypsin, porcine, from Sigma.

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ADDENDUM

After the pioneering work of Ross Harrison, other researchers took his lead and started to experiment with cell culture. Many of them were particularly interested in culturing developing nerve tissues, and in looking for the substances and media that would encourage neurite outgrowth. Burrows, for example, (Burrows 1911), was the first to show growth of nerve fibres from a warm blooded organism *in vitro*. He used the dorsal root ganglia of chicken embryos of sixty hours of incubation, and cultured them in hanging drops of clotted chicken plasma. After him, Lewis and Lewis (1912) produced a paper in which they described the culture of sympathetic nerves from the intestine of chick embryos. In this case, they used a number of saline solutions as culture media, and were able to observe sustained neurite outgrowth for up to forty eight hours. This was an important step towards the development of defined growth media. Another pioneering researcher in the field was Ingebrigtsen (1913), who observed the growth of nerve fibres from pieces of the central nervous system of rabbits, cats, and dogs, in clotted plasma drops. After the work of these people, developments in the field came slowly but steadily. The main area of improvement was the definition of the growth media, and the isolation of the factors that would encourage nerve cell survival and neurite growth in the media. In this way, Martinovic (1931) used cerebrospinal fluid in his medium, and noted that this would cause the cells to migrate on the glass surface. Murray and Stout (1947) cultured human sympathetic ganglia in media composed of foetal or

placental serum superimposed on a plasma clot, and managed to maintain the cultures alive for periods of months. Pomerat (1951) also induced adult CNS tissue to survive for months in media consisting of neoplastic ascitic fluid and embryo extract. It was also Pomerat (Pomerat and Costero, 1956) who introduced a supplement of glucose to the medium. Shortly after, Levi-Montalcini (1956, 1960) isolated nerve growth factor from mouse salivary glands. From this time on, the introduction of antibiotics in the media, and improved sterile methods, made cell culture a much easier technique, and the field advanced at a faster rate as more and more investigators started using it.

There are two additional pieces of work that greatly advanced our understanding of how developing nerve fibres reach their destination, and which are worth mentioning here. One is the work that Paul Weiss conducted during the 30's and 40's, and reviewed in 1945 (Weiss 1945). He cultured nerve tissues in media composed of serum or plasma and embryo extract. In these cultures, he introduced fibres of various materials, glass, textiles, synthetic resins, or well defined scratches on mica substrata. He observed that the outgrowing nerve fibres were oriented by these structures, studied the way in which this phenomenon happened, and defined the term contact guidance to describe it.

The other investigation I referred to above was by R. W. Sperry (1951). He studied the mechanisms of development of the retinotectal projection, by watching the effects of surgically rotating the eyes of newt embryos through different angles. He arrived at the conclusion that each nerve cell has the information it needs to reach its appropriate target, regardless of its starting position.

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